MECHANISMS OF ESTROGEN-DEPENDENT DENDROGENESIS IN MOTONEURONS OF THE SPINAL NUCLEUS OF THE BULBOCAVERNOSUS

Lauren Marie Rudolph

Submitted to the faculty of the University Graduate School

in partial fulfillment of the requirements for the degree

Doctor of Philosophy

in the Neuroscience Program and the Department of Psychological and Brain Sciences,

Indiana University

July 2014

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree Doctor of Philosophy

Doctoral Committee

_____

Dale R. Sengelaub, PhD, co-chair

_____

Gregory E. Demas, PhD, co-chair

_____

G. Troy Smith, PhD, member

_____

Cara L. Wellman, PhD, member

June 13, 2014

This thesis is dedicated to my wonderful parents, Dr. Rhys and Laura Rudolph, who taught me to think like a scientist, be curious about the world around me, and never take myself too seriously.

Acknowledgements

In short, these past five years have been a blast. Of course it is easy to have a fun, successful graduate career when you have an advisor like Dale Sengelaub. Dale, you are an outstanding scientist, a second-to-none mentor, and a loyal and valued friend. I have learned an enormous amount from you: From the ins and outs of protocols and science, to how proper advising should occur, to education about the far superior traditional grip drumming, plate of shrimp moments, and the *actual* noise that lasers make. You are one of the most loyal, fun, creative, and enthusiastic people I have had the pleasure of getting to know, and it has been an absolute joy and privilege to be your graduate student for the past five years. Thank you for believing in me, teaching me, supporting me, and being my number one cheerleader throughout graduate school. I am honored to be in the ranks of Sengelaub Lab graduates, and aspire to follow in your footsteps as a successful mentor and scientist. Of course I am sad my graduate training has to end, but fortunately the completion of the PhD does not mark the end of DLBTs, VOS beer, BAMFACTS, and all the other classic Lobster shenanigans. One could argue the fun is just beginning.

Thank you to my committee members, Cara Wellman, Greg Demas, and Troy Smith, for your valuable expertise throughout my graduate career. Your thoughtful editing and insightful comments have always resulted in an improved final product, whether that was a manuscript, presentation, or list of adult beverages for a CISAB conference reception. I am so thankful I had the opportunity to get to know you and learn from you over the past five years.

Thank you to Rhys and Laura Rudolph, my parents, for your support during this experience. I am continually humbled by your demonstrations of patience and love for me. I am so fortunate to have you as parents and I hope you know how much I deeply and truly appreciate you and love you. Thank you to my sister, Kathryn Diekhoff, and brother-in-law Mike Diekhoff for your friendship, love, and support over the years. I am grateful we had the opportunity to spend time together in Bloomington, and of course appreciate your adherence to the “no frat party” rule at Hotel Rudy.

To Dr. Spencer and Mary Goodson: I am so grateful I was able to get to know you over the last five years. I have thoroughly enjoyed having you as my Bloomington family (and I know Lena has enjoyed all those walks), and will miss sharing meals and visits with you. Thank you for opening your home to me and for including me in your lives.

Thank you, Mollee Farrell, for being my Dara Lab partner in crime during graduate school. From “Tream Brian” to silver SUVs at Three Floyd’s to SfN, we have created so many fun memories. Thank you for always being game for an adventure and willing to laugh along with me through some ridiculous situations like the N650 lunch with she-who-must-not-be-named. Mark Peterson and Josh de Leeuw, my science and volleyball pals, or “double major” friends: Thank you for making science jokes on the volleyball court (Kinky Sets for life!) and athletic jokes in the lab (FYI: birds and robots are inferior model species). Thank you to the rest of the volleyball crew: Jeff Chou, Bret Guzik, Nate Han, Moe Pazos, Melissa Toups, Jason Wesson, Martin Wolfger, and Zalmai Yawar, for the memories on and off the court. I will continue to “watch short” and try to refrain from acting like a village person in California and beyond.

Thank you to CISAB and the CTRD, for their support of my research during graduate school. Thanks to both the CISAB and CTRD community for providing me with the opportunity to share my research with a wider audience, and to be exposed to so many fascinating people with a variety of scientific backgrounds. I am proud to call myself a member of both organizations, and have grown both personally and scientifically from my affiliation with them. A special thanks to Linda Summers for making my involvement in the CISAB and the CTRD a fun, easy-to-navigate experience.

I am grateful to Dr. Rose Stewart for her expert ELISA skills. Thank you for your patience and expertise you shared with me while in the lab. It was a joy to spend time with you, whether creating standard curves or challenging each other at Quiz Up.

I have been fortunate to meet some truly unique, genuinely fun people while in Bloomington. To all of you, thank you for being a part of my life, and for allowing me to be a part of yours. I will treasure the times we have shared, and I promise to keep in touch, and create more fun-filled memories in the future.

Lauren Marie Rudolph

MECHANISMS OF ESTROGEN-DEPENDENT DENDROGENESIS IN MOTONEURONS OF THE SPINAL NUCLEUS OF THE BULBOCAVERNOSUS

Early hormone exposure is required for the development of many neural structures across a variety of species. The spinal nucleus of the bulbocavernosus (SNB), a motor nucleus that controls erections and ejaculations in male rats, requires gonadal hormones for typical development. While the masculinization of many features of the SNB neuromuscular system is androgen-dependent, dendrogenesis requires estrogens during a developmentally restricted critical period. This estrogen-dependent SNB dendrite growth is mediated by estrogen receptors (ERs) in the SNB target musculature (the bulbocavernosus, BC). I have demonstrated that ERα expression in BC extra-muscle fiber cells defines the critical period for estrogen-dependent SNB dendrite growth, and estradiol treatment during ERα expression in these extra-muscle fiber cells fully masculinizes SNB dendrites. Further, BC ERα expression is sensitive to gonadal hormones: Castration dramatically upregulates the density of extra-muscle fiber cell ERα. Brief estradiol treatment during heightened ERα expression results in rapid, robust dendritic growth, suggesting that the estrogen sensitivity of SNB dendrites is determined by ERα levels in the target musculature. I have shown that the castration-induced increase of ERα in the BC extends the critical period for estrogen-dependent SNB dendrogenesis, the first demonstration that SNB dendrites respond to estrogens outside the early postnatal period. I have shown that brain-derived neurotrophic factor (BDNF) is upregulated in the SNB target muscle following early postnatal castration, but immunolabeling for BDNF in SNB somata does not change. In both cases, estradiol treatment does not affect BDNF levels, demonstrating that while there may be a role for BDNF in early dendrite growth, estrogen-dependent dendrogenesis is not mediated by BDNF. Finally, I have shown that the ERα-expressing extra-muscle fiber cells in the BC are not satellite cells, and the specific cell type mediating early estrogen-dependent dendritic growth remains unknown. Together, these results elucidate the mechanisms of estrogen-dependent dendrite development in the SNB of male rats, establish the basis for the estrogen-sensitive critical period, and demonstrate the plasticity of estrogen-mediated dendrogenesis in the SNB.

Table of Contents

CHAPTER 1: Introduction

The lumbar spinal cord of rats contains a sexually dimorphic nucleus, the spinal nucleus of the bulbocavernosus (SNB), also known as the dorsomedial nucleus (DM; Schrøder, 1980). In male rats, the SNB consists of ~200 motoneurons that innervate the perineal muscles bulbocavernosus (BC), levator ani (LA) and the anal sphincter (Breedlove and Arnold, 1980; Schrøder, 1980; McKenna and Nadelhaft, 1986; Fig. 1.1). The BC/LA muscles attach to the base of the penis, and are necessary for successful copulation and insemination (Sachs, 1982; Hart and Melese-D'Hospital, 1983). In female rats, the perineal musculature is absent or greatly reduced and the SNB consists of ~60 motoneurons that predominantly innervate the external anal sphincter (Hayes, 1965; Čihák et al., 1970; McKenna and Nadelhaft, 1986; Tobin and Joubert, 1991). The morphology of SNB motoneurons is also sexually dimorphic, and SNB somata of adult females are typically half as large as those of adult males (Breedlove and Arnold, 1981; McKenna and Nadelhaft, 1986).

The dimorphisms in the SNB neuromuscular system result from the actions of gonadal steroids, operating both developmentally and in adulthood. During perinatal development, androgens are responsible for the prevention of normally occurring motoneuron death (Nordeen et al., 1985), somal growth (Lee et al., 1989; Goldstein et al., 1990; Goldstein and Sengelaub, 1992), perineal musculature retention (Čihák et al., 1970), and neuromuscular synapse elimination (Jordan et al., 1989a; Jordan et al., 1989b). Conversion of testosterone to estrogenic metabolites does not appear to be involved in this regulation, and treatment with estradiol has no effect on SNB motoneuron survival (Breedlove et al., 1982; Goldstein et al., 1990), somal growth (Breedlove et al., 1982; Goldstein and Sengelaub, 1994; Burke et al., 1997; Hebbeler et al., 2002), target muscle retention (Breedlove et al., 1982; Burke et al., 1997), or neuromuscular synapse elimination (Jordan et al., 1995).

Conversely, dendritic development in the SNB is regulated by both androgens and estrogens. During normal development, SNB dendrites grow profusely during the first 4 postnatal weeks (Goldstein et al., 1990). This growth is steroid-dependent: In males castrated 7 days after birth, dendrites fail to grow beyond their precastration lengths, whereas dendritic lengths of

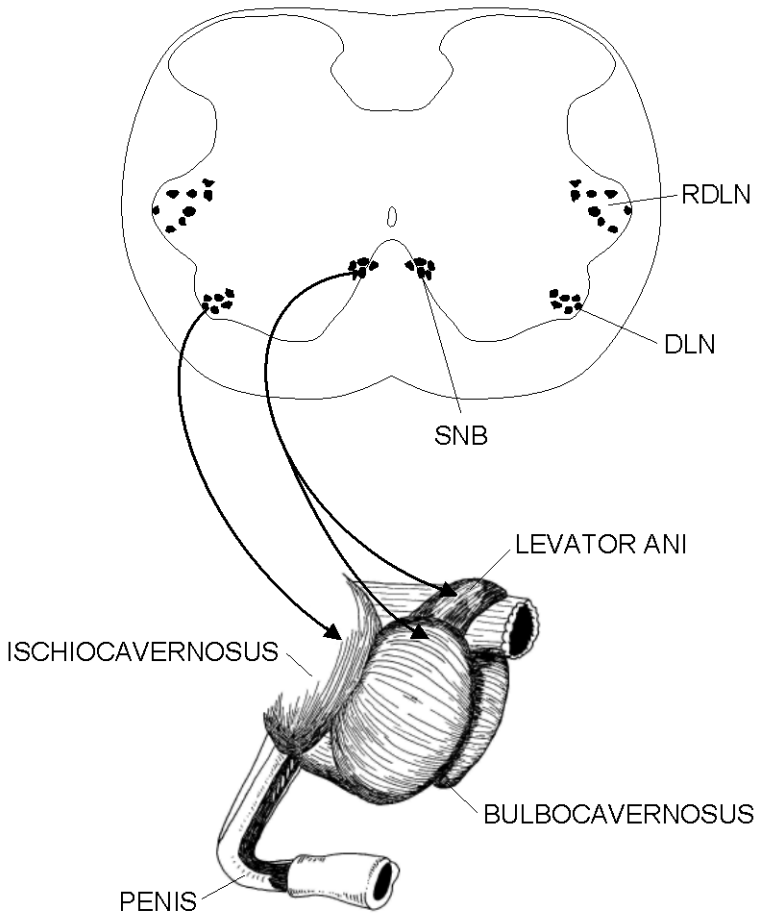

Figure 1.1. Schematic transverse section of lumbar spinal cord of the male rat. Motoneurons of the spinal nucleus of the bulbocavernosus (SNB) are located medially. These motoneurons innervate the bulbocavernosus and levator ani (BC/LA) muscles that attach to the base of the penis and are involved in erections in ejaculation. Other major motor nuclei present in lumbar spinal cord include the sexually dimorphic dorsolateral nucleus (DLN) that innervates the ischiocavernosus muscle, and the sexually monomorphic retrodorsolateral nucleus (RDLN) that innervates the extensor digitorum longus muscle of the foot.

castrated males receiving testosterone replacement are not different from those of intact males by 4 weeks of age (Goldstein et al., 1990). Treatment with either of testosterone's metabolites, dihydrotestosterone or estradiol, partially supports dendritic growth (Goldstein and Sengelaub 1994), but combined treatment with both metabolites is as effective as testosterone in fully supporting dendritic growth (Goldstein and Sengelaub, 1994). Estrogenic support of postnatal dendritic growth appears to be mediated by estrogen action at the neuromuscular periphery, as local blockade of estrogen receptors (ERs) with tamoxifen implants at the SNB target musculature during the period of dendritic growth results in significantly decreased dendritic lengths, but placing identical implants at the scapula has no effect on SNB dendritic growth (Nowacek and Sengelaub, 2006). Conversely, treatment with estradiol muscle implants in castrates supports dendritic growth through postnatal day 28, demonstrating that estradiol action at the periphery during the early postnatal period is critical for SNB dendrite growth (Nowacek and Sengelaub, 2006). This estrogenic support of SNB dendrites is transient, restricted to the early postnatal period: by the seventh week of age, dendritic lengths in estradiol-treated castrates are not different from those in oil-treated castrates, and are shorter than those of castrates treated with either testosterone or dihydrotestosterone (Goldstein and Sengelaub, 1994). In adulthood, SNB dendritic lengths in castrates can be fully maintained with dihydrotestosterone or testosterone, but estradiol is completely ineffective in supporting dendritic morphology (Verhovshek et al., 2010b). Chapter 2 of this dissertation addresses why estrogen-dependent dendrite growth mediated through ERs in the SNB target muscle is transient, by assessing ERα immunolabeling in the BC muscle during the estrogen-sensitive period, and outside of it. Experiments from Chapter 2 also address the timing of estrogen treatment in relation to ERα levels in the BC muscle, and how this relates to estrogen-dependent dendrite growth during the early postnatal period. Chapters 3 and 4 build upon the results of Chapter 2 experiments and assess the influence of gonadal hormones on ERα in the BC muscle, and how changes in peripheral ERα density affects the potential for SNB motoneuron dendrites to grow in response to estrogen treatment, both during and outside of the critical period for estrogen-dependent dendrogenesis that occurs during early postnatal life in male rats.

The early chapters of this thesis are focused on elucidating the developmental profile of ERα in the SNB target muscle and how this expression profile relates to the critical period for estrogen-dependent SNB dendrogenesis; the later Chapters are focused on the potential molecular mechanisms that control estrogen-dependent SNB dendrite growth. The experiment in Chapter 5 assesses ERα expression in a non-muscle fiber cell type in the SNB target muscle, to better understand the nature of ERα expression in the SNB target muscle during the early postnatal period. The goal of Chapter 6 is to further elucidate the molecular mechanisms of SNB dendrite growth in response to estrogens during early development. We do not know the pathways downstream of ER activation in the BC muscle that control estrogen-dependent dendrite growth. However, the molecular mechanisms of androgen-dependent influences on SNB dendrites in adulthood have been investigated and involve brain-derived neurotrophic factor (BDNF), so it is possible that similar mechanisms underlying the maintenance of androgen-dependent of adult SNB motoneuron morphology also control estrogen-dependent changes in SNB motoneurons during development.

Gonadal hormone regulation of BDNF and BDNF-androgen interactions maintain SNB morphology in adulthood (for review, see Verhovshek et al., 2012). BDNF mRNA and protein are decreased in SNB somata and proximal dendrites after castration in adulthood, and testosterone treatment restores this castration-induced reduction in SNB motoneuron BDNF (Ottem et al., 2007; Verhovshek et al., 2010a). Conversely, in the SNB target musculature, adult castration results in an increase in BDNF expression, and testosterone treatment returns BDNF to its precastration levels (Verhovshek et al., 2010a). The castration-induced increase in muscle BDNF and decrease in SNB motoneuron BDNF could reflect a decrease in retrograde transport of BDNF from the BC/LA to SNB motoneurons or an increase in peripheral BDNF production in the absence of gonadal hormones. Regardless of the mechanism, the castration-induced increase in muscle BDNF coincides with SNB muscle, somal, and dendritic atrophy. Given that the SNB target musculature is the site of action for the maintenance of SNB morphology by gonadal hormones, and a site of androgenic regulation of BDNF in SNB somata, these data suggest that heightened BDNF levels in the muscle could be mediating the castration-induced atrophy in the

SNB system. Blockade of ARs at the SNB target muscle reduces BDNF expression in motoneurons, and local testosterone treatment restores the castration-induced decrease in BDNF levels in SNB somata (Verhovshek and Sengelaub, 2013).

Interrupting BDNF signaling using trkB IgG affects SNB motoneuron morphology, and appears to promote dendrogenesis. SNB dendritic arbors of both castrated and intact males hypertrophy when BDNF is blocked, resulting in dendrite lengths that are longer than those of intact control animals. Similarly, blocking BDNF signaling prevents castration-induced SNB somal atrophy (Verhovshek and Sengelaub, 2010b). Additionally, BDNF has also been implicated in the perinatal masculinization of SNB motoneuron number. Treating animals with trkB IgG blocks the androgen-induced prevention of SNB cell death, suggesting that androgen-BDNF interactions also play a role in hormone-mediated masculinization of the SNB during early development (Xu et al., 2001). Together, these data demonstrate that BDNF expression in SNB motoneurons and their target musculature are regulated by gonadal hormones, and BDNF signaling plays a role in the development and maintenance of SNB morphology.

In addition to BDNF as a critical player in SNB morphology in adulthood, there are examples of estrogen-induced BDNF regulated of morphology across the neuraxis. The *Bdnf* gene contains an estrogen response element (Sohrabji et al., 1995), providing a direct mechanism for estrogenic regulation of BDNF transcription. In neonatal mice, estradiol treatment increases BDNF levels and stimulates dendritic spino- and synaptogenesis in the cerebellar Purkinje cells (Sasahara et al., 2007). Estradiol-induced upregulation of BDNF is blocked by the ER antagonist tamoxifen, demonstrating that ERs mediate the estrogenic regulation of BDNF in these cells. Estradiol has been shown to promote dendrite growth by either increasing (Krizsan-Agbas et al.,2003) or decreasing (Murphy et al., 1998) BDNF levels, demonstrating that estradiol-dependent dendrite growth is mediated by changes in BDNF signaling in other systems and BDNF could be an estrogen-regulated molecule that affects developmental dendrite growth in the SNB. During early postnatal development, castration results in an increase in BDNF protein in the hippocampi of male rats, and estrogen treatment of castrates decreases BDNF to precastration levels (Solum and Handa, 2002). These data suggest that estrogen acts to repress

BDNF expression during early development in rat hippocampus both *in vitro* and *in vivo*, potentially through receptor-mediated pathway, as ERα and BDNF are coexpressed in these hippocampal neurons (Solum and Handa, 2002). This suggests ERα is the potential site of action for estrogenic regulation of BDNF in these neurons, as ERβ-BDNF colocalization does not occur in these cells. These data suggest that estrogen can regulate BDNF in the same manner as androgens in the SNB system, and demonstrate that estrogen-dependent dendrite growth could be mediated by changes in muscle BDNF. The experiments of Chapter 6 are designed to determine if BDNF is involved in estrogen-dependent SNB dendrite growth during the early postnatal period, using immunohistochemistry and enzyme-linked immunoassay (ELISA) techniques to measure BDNF in both the peripheral and central components of the SNB neuromuscular system after castration and estradiol treatment.

Together, the experiments of this dissertation focus on understanding how estrogen-dependent SNB dendrite growth occurs, and why there is a developmental critical period for this form of SNB dendrogenesis, and what mechanisms underlie this transient, estrogen-mediated dendrite growth in a typically estrogen-insensitive neuromuscular system. The following experiments and resultant data describe mechanisms of estrogen-dependent dendritic growth in the SNB neuromuscular system and provide insight into how hormone-directed sexual differentiation proceeds in sexually dimorphic structures within the mammalian nervous system.

CHAPTER 2: Rudolph, L.M. & Sengelaub, D.R. (2013). Critical period for estrogen-dependent motoneuron dendrite growth is coincident with ERα expression in target musculature. *Developmental Neurobiology.* 73(1): 72-84.

BACKGROUND

Because estrogen-dependent SNB dendrite growth is transient and mediated by estrogen action at ERs in the target musculature, we hypothesized that the developmentally restricted estrogen-dependent SNB dendrite growth was mediated by a change in ER expression at the target musculature. In Experiment 1, we used immunohistochemistry to assess ERα labeling in the BC muscle throughout the period of estrogen-dependent SNB dendrogenesis, and later in postnatal development and in adulthood, when SNB dendrites are insensitive to estrogens. Basal lamina immunolabeling was quantified in the same ERα-labeled tissue sections to determine the location of ERα label in relation to muscle fibers (identified by basal lamina immunolabeling).

Experiment 1

MATERIALS AND METHODS

Animals. Untimed pregnant rat dams (Sprague-Dawley, Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle, with unlimited access to food and water. When pregnant females gave birth (day of birth = P1), pups were sexed and litters culled when necessary, with preference for males over females. Males were killed weekly from P7 to P49, and in adulthood (approximately 100 d old); overall $n = 40$ (P7, $n = 6$; P14, $n = 6$; P21, $n = 6$; P28, $n = 6$; P35, $n = 4$; P42, $n = 3$; P49, $n = 3$; adult, $n = 6$). All procedures were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Bloomington Institutional Animal Care and Use Committee.

Immunohistochemistry. Animals were weighed, overdosed with urethane (0.25 g/100 g of body weight), and perfused transcardially with saline followed by cold 4% paraformaldehyde in 0.1M phosphate buffer (pH = 7.4). The BC/LA muscles were removed and postfixed in the same fixative for 24 h and transferred to 30% sucrose in 0.1M phosphate buffer for a minimum of 24 h of cryoprotection. The BC/LA was cut horizontally into 12 µm sections on a cryostat at -16°C. Sections were thaw-mounted onto gelatin-coated slides, and stored at -16°C. For immunohistochemical processing, slides were brought to room temperature, removed from the slide boxes, placed in a glass staining rack and rinsed 3 x 5 min in phosphate buffered saline

(PBS, pH 7.4). After rinsing, a hydrophobic border (Super Pap Pen, Ted Pella, Inc., Redding, CA) was created around each tissue section, and the first incubation did not occur until the border had dried (about 3 min). All incubations occurred in a humidified chamber and were performed by pipetting 200 µl of solution onto each section.

Sections were incubated for 45 min at room temperature in blocking solution containing 10% normal goat serum (NGS; Vector Laboratories, Inc., Burlingame, CA) and 0.2% Triton X-100 in PBS. Sections were then incubated for 48 h at 4ºC in 4% NGS in PBS containing an antibody directed against the last 14 amino acids of the rat ERα (C1355, 1:1000; Millipore, Temecula, CA). After primary ERα incubation, sections were rinsed 3 x 5 times in PBS and incubated for 2 h at room temperature in 4% NGS in PBS containing a conjugated secondary antibody (goat anti-rabbit Alexa Fluor 488 $F(ab')_2$ fragments (H+L), 1:200; Invitrogen, Eugene, OR). Sections were then rinsed 3 x 5 min in PBS, and incubated in a 4% NGS in PBS solution containing a mouse monoclonal antibody for basal lamina (D18 supernatant, 1:50; Developmental Studies Hybridoma Bank, Iowa City, IA) for 12-18 h at 4°C. Following this incubation, sections were rinsed 3 x 5 times in PBS and incubated for 2 h at room temperature in a secondary antibody (goat anti-mouse IgG-TRITC, 1:200; Sigma-Aldrich, Oakville, Ontario, Canada). Sections were then rinsed 3 x 5 min in PBS, briefly air dried, and coverslipped with aqueous mounting fluid (Vectashield HardSet Mounting Medium; Vector Laboratories, Inc.). After slides dried for 5 min at room temperature, they were sealed with nail polish, dried for 10 min at room temperature, laid flat to dry overnight at 4°C, and then stored at 4°C. To control for nonspecific staining, control sections incubated without both primary antibodies were generated and demonstrated no immunostaining.

Microscopic Analysis. Because all of our previous developmental work on SNB motoneuron dendritic growth, hormone sensitivity, and critical period effects were done in BC-projecting motoneurons, our analysis was restricted to the BC muscle. After slides were brought to room temperature, sections of muscle were first viewed at 150-350X magnification under epifluorescent illumination to visualize basal lamina staining. The BC is a relatively complex muscle, with muscle fibers traveling in several different directions, resulting in fields containing both cross- and longitudinally sectioned muscle fibers (Fargo et al., 2003); fields containing large numbers of

basal lamina-stained muscle fibers in cross-section were selected for analysis. Because cross-sectional muscle fiber size varied across ages, variable magnifications and sampling frames were employed. After locating areas of tissue that contained sufficient numbers of cross-sectioned muscle fibers, magnification was increased until the field of view contained a minimum of 14 fibers, resulting in a final magnification of at least 1450X. Using Stereo Investigator (MBF Bioscience, Inc., Williston, VT), muscle fibers were sampled systematically, by either analyzing all fibers in a field of view or superimposing a grid on the tissue image (e.g., 100 μm x 100 μm square frame) and sampling all fibers within the frame.

To quantify ERα immunostaining, muscle fibers were visualized under FITC fluorescence and images were captured for subsequent analysis. Areas that appeared to be labeled relative to background (see below) were identified as either in-fiber or extra-fiber depending on the location of ERα label relative to the basal lamina stain. One field per section and 1-3 sections per animal were analyzed; overall, an average 69.40 muscle fibers per animal were traced. For each analyzed tissue section, the mean and standard deviation of muscle fiber FITC luminance values were calculated and a minimum labeling criterion of the mean plus two standard deviations was established for each section. Cells that exceeded this criterion were identified as ERα-positive and were categorized as either ERα-positive muscle fibers (if surrounded by a basal lamina) or ERα-positive extra-muscle fiber cells. Because muscle fiber area increases with age (potentially decreasing the density of immunolabeled muscle fibers), we sampled equivalent numbers of muscle fibers across ages and expressed ERα-positive muscle fibers as a percentage of the sample. ERα-positive extra-muscle fiber cells were identified only on the basis of ERα labeling, so these numbers were expressed as a density per unit area. The percentage of ERα-positive muscle fibers and density of ERα-positive extra-muscle fiber cells per section were analyzed using one-way ANOVAs with Fisher's LSD post-hoc tests. Differences were considered significant if $p < 0.05$.

RESULTS

ERα immunolabeling was present in the BC muscle at all ages examined. Immunolabeling in muscle fibers was not restricted to nuclei, suggesting cytosolic localization. ERα immunolabeling was categorized as either ERα-positive muscle fibers (label contained within the basal lamina stain) or ERα-positive extra-muscle fiber cells (label outside basal lamina stain; Fig. 2.1). The average number of muscle fibers analyzed per animal did not differ across ages [$F(7, 32) = 1.21$, n.s.]. For each animal, the number of labeled muscle fibers was expressed as a percentage of total muscle fibers sampled. The percentage of ERα-positive muscle fibers did not vary with age [mean = 7.01% ± 0.89; $F(7, 32) = 2.20$, n.s.].

There was a significant main effect of age on the density of ERα-positive extra-muscle fiber cells [$F(7, 32) = 2.32$, $p < 0.05$; Fig. 2.2]. ERα-positive extra-muscle fiber cells were abundant at P7 (209.3 ± 102.8 per mm^2) and P14 (285.2 ± 115.1 per mm^2) compared to adult levels (LSDs, ps < 0.05). At P21, the density of ERα-positive extra-muscle fiber cells was decreased by 38%, and continued to decline into adulthood (0.00 ± 0.0 per mm^2). The densities of ERα-positive extra-muscle fiber cells after P21 did not differ significantly from adult levels (LSDs, n.s.).

Experiment 2

In Experiment 1, we found that the density of ERα-positive BC extra-muscle fiber cells decreased significantly after P21. Based on these results, we hypothesized that BC extra-muscle fiber cells are the site of estrogenic action in supporting SNB dendrite growth, and that the transient expression of ERα within BC extra-muscle fiber cells during the early postnatal period could define the critical period for estrogen-dependent SNB dendrite growth. To test this hypothesis, in Experiment 2, we castrated males and treated them with estradiol via BC muscle implants from P7 to P21, and assessed SNB dendritic morphology at P28, the day at which SNB dendrites normally reach maximal length (Goldstein et al., 1990).

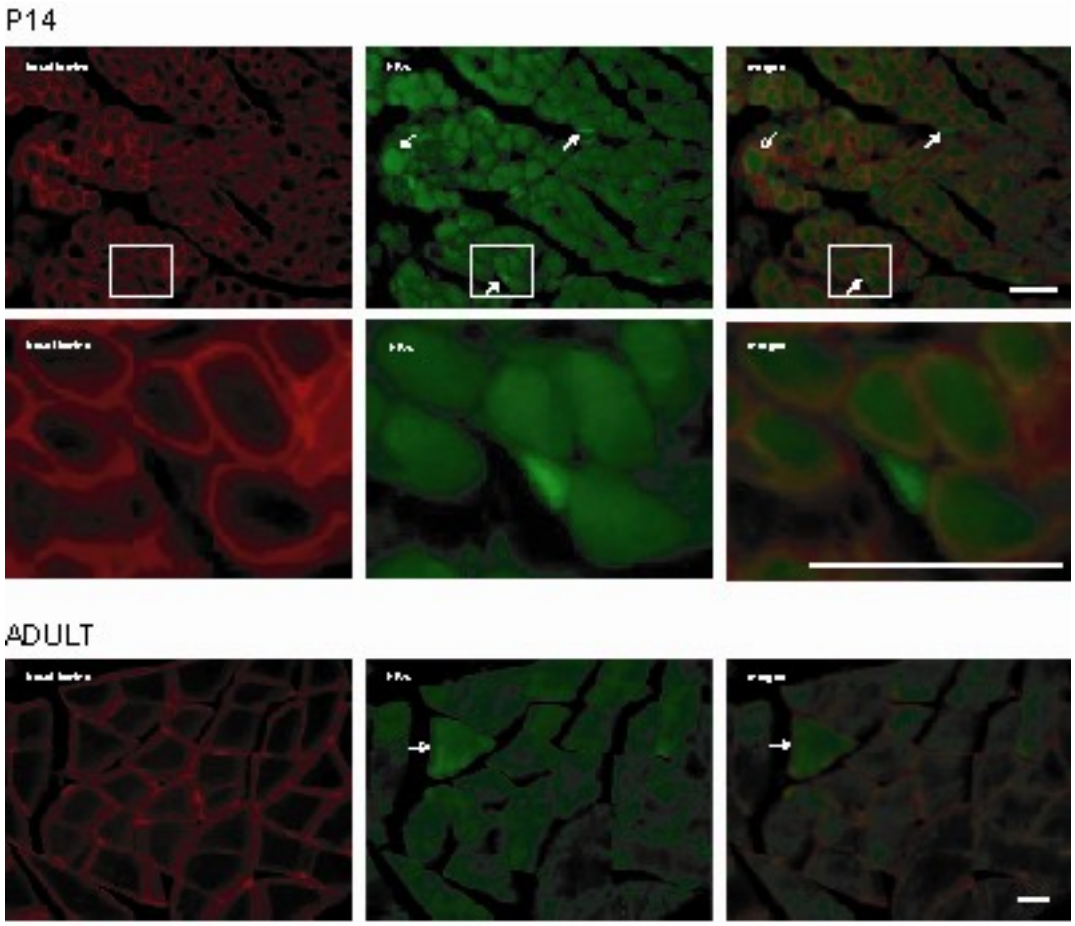

Figure 2.1. Digital light micrographs of cross-sections of BC muscle in P14 (top row) with higher magnification views of indicated areas (middle row), and adult males (bottom row). Each row shows the same tissue section viewed under different epifluorescent illumination, demonstrating immunolabeling for basal lamina only (red; left), ERα only (green; middle), and the merged image showing combined basal lamina and ERα immunolabeling (right). Arrows indicate ERα-positive muscle fibers (open arrows) and ERα-positive extra-muscle fiber cells (white arrows); note the absence of basal lamina staining of the indicated extra-muscle fiber cell in the middle row. Scale bars = 25 μm.

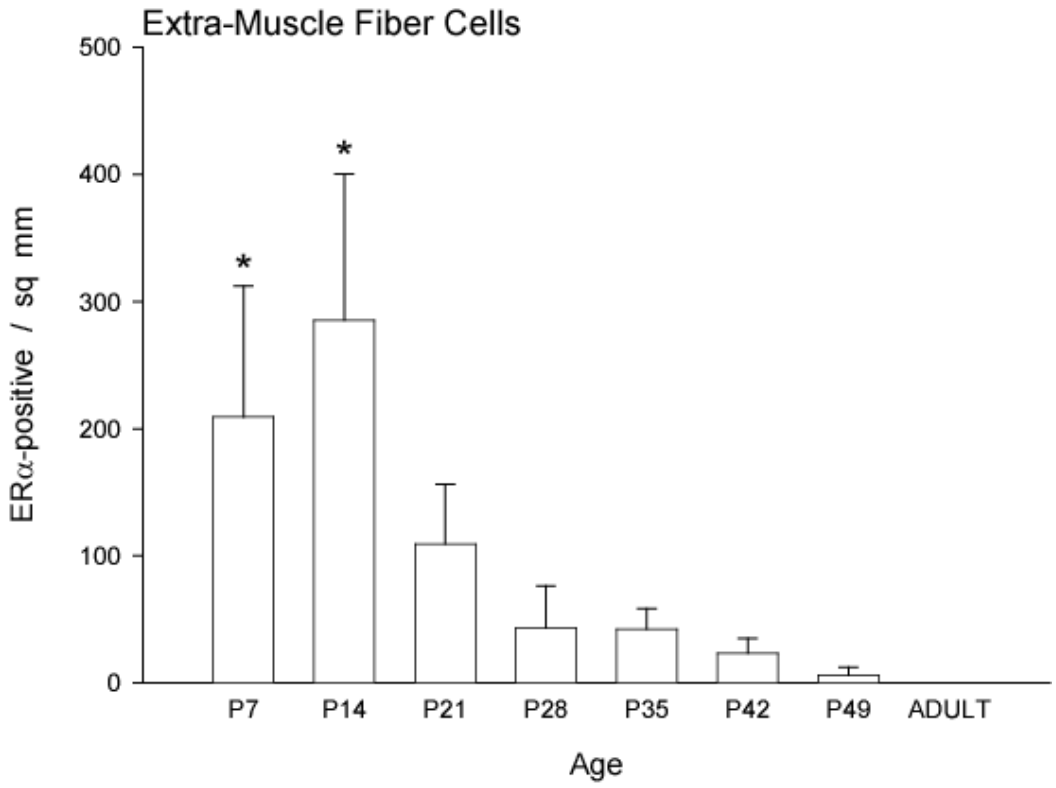

Figure 2.2. The number of ERα-positive extra-muscle fiber cells per mm^2 in the BC at P7, P14, P21, P28, P35, P42, 49, and in adulthood. Bar heights represent means ± SEM for three to six animals per group. Asterisk denotes significantly different from adult animals.

MATERIALS AND METHODS

Animals. Untimed pregnant rat dams (Sprague-Dawley; Harlan) were maintained on a 12-h light, 12-h dark cycle, with free access to food and water. Litters were culled to eight pups when necessary, retaining males preferentially. Males were bilaterally castrated under isoflurane anesthesia at P7, and were given estradiol implants (see below) at the left BC muscle or were untreated (n = 7). In estradiol-treated castrated males, some implants were removed at P21 (n = 6), and in other animals, implants remained until P28 (n = 5). Age-matched intact control males (n = 5) were also used (overall n = 23). All procedures were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Bloomington Institutional Animal Care and Use Committee.

Implants. To restrict estradiol treatment to the target musculature, we used our previous method (Nowacek and Sengelaub, 2006) to deliver hormones locally to the BC muscle. This treatment produces alterations in motoneuron morphology that can be directly ascribed to local rather than systemic effects; furthermore, the treatment has no effect on the normal development of either the BC muscle or SNB motoneurons, and no effect on dendritic labeling or tracer transport (Nowacek and Sengelaub, 2006). Small (0.85 mm^3) implants impregnated with estradiol (Steraloids, Newport, RI) were constructed by mixing crystalline estradiol with Silastic adhesive (Dow-Corning, Auburn, MI) and compressing the mixture between two glass slides (lightly greased with petroleum jelly) separated by a spacer (0.85 mm thick), and allowed to cure. The cured Silastic was cut into 1 x 1 mm pieces (total volume 0.85 mm^3) and coated on five sides with acrylic. The amount of estradiol in each implant (0.1 mg) was identical to that used previously; this dosage does not produce systemic effects, and supports SNB dendritic growth.

Histochemistry. Horseradish peroxidase conjugated to the cholera toxin β subunit (BHRP; List Biological, Campbell, CA) was used to retrogradely label SNB motoneurons innervating the BC muscle. Previous studies have demonstrated that BHRP labeling permits sensitive detection and quantitative analysis of SNB somal and dendritic morphologies (Kurz et al., 1986; Goldstein and Sengelaub, 1990; Hebbeler and Sengelaub, 2003). SNB motoneuron morphology was examined at P28 (when SNB dendritic length is normally maximal; Goldstein et al., 1990). At P26, animals

were anesthetized with isoflurane, the perineal muscles exposed, and BHRP unilaterally injected (0.5 µl; 0.2% solution) into the left BC muscle, the same muscle for those males that received muscle implants. Forty-eight h after BHRP injection, a period that ensures optimal labeling of SNB motoneurons (Kurz et al., 1986; Goldstein et al., 1990), animals were overdosed with urethane (0.25 g/100 g body weight) and perfused intracardially with saline followed by cold 1% paraformaldehyde/1.25% glutaraldehyde fixative. Lumbar cords from all animals were removed, postfixed in the same fixative for 5 h, and transferred to sucrose phosphate buffer (10% w/v, pH = 7.4) overnight for cryoprotection. Spinal cords were then embedded in gelatin, frozen, and sectioned transversely at 40 µm; all sections were collected into four alternate series. For visualization of BHRP, the tissue was immediately reacted using a modified tetramethyl benzidine protocol (TMB; Mesulam, 1982), mounted on gelatin-coated slides, counterstained with thionin, and cover-slipped with Permount. BC/LA muscles were removed at perfusion and weighed to evaluate potential treatment effects on gross muscle development.

Motoneuron Somata. The number of BHRP-filled motoneurons was assessed in all sections through the entire rostrocaudal extent of the SNB for all animals. Counts of labeled motoneurons in the SNB were made under brightfield illumination, where somata and nuclei could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed. Estimates of the total number of labeled SNB motoneurons were obtained using the optical dissector method outlined by Coggeshall (1992) and a procedure similar to that of West and Gunderson (1990). This method yields an unbiased count of SNB motoneurons (Raouf et al., 2000). Counts were made at 500X; motoneuron somata could be easily visualized in multiple focal planes. BHRP-labeled motoneurons were counted as their somata first appeared in focus while focusing through the *z* axis, and labeled somata in the first focal plane (i.e., “tops”) were not counted. For each animal, counts were derived from sections spaced at 160 µm intervals uniformly distributed through the entire rostrocaudal extent of the SNB. Within each section, all labeled somata within the SNB were counted. Estimates of the total number of labeled SNB motoneurons were then obtained by correcting for percentage of the tissue sampled.

The cross-sectional soma area of BHRP-labeled motoneurons was measured in an average of 21.8 motoneurons for each animal using a video-based morphometry system (Stereo Investigator; MBF Bioscience, Inc.) at a final magnification of 1350X. Soma areas within each animal were averaged for statistical analysis. The optical density of labeled somata was also measured under brightfield illumination to confirm equivalence of BHRP labeling density.

Dendritic Length. For each animal, dendritic lengths in a single representative set of alternate sections were measured under darkfield illumination. Beginning with the first section in which BHRP-labeled fibers were present, labeling through the entire rostrocaudal extent of the SNB dendritic field was assessed in every other section (320 μm apart) in three dimensions using a computer-based morphometry system (Neurolucida; MBF Bioscience, Inc.; final magnification 250X) to yield both composite illustrations of the arbor and measurements of individual fiber lengths. All BHRP-labeled fibers were drawn regardless of location, size, or contiguity with labeled cell bodies to ensure a complete assessment of dendritic length. Because the entire rostrocaudal range of the SNB dendritic field in each animal was sampled, this method allows for a complete assessment of SNB dendrites in both the transverse and horizontal planes. Average dendritic arbor per labeled motoneuron was estimated by summing the measured dendritic lengths of the series of sections, multiplying by two to correct for sampling, then dividing by the total number of labeled motoneurons in that series. This method does not attempt to assess the actual total dendritic length of labeled motoneurons (Kurz et al., 1991), but has been shown to be a sensitive and reliable indicator of changes in dendritic morphology in normal development (Goldstein et al., 1990), after hormonal or surgical manipulation (Kurz et al., 1986; Goldstein et al., 1990; Kurz et al., 1991; Goldstein and Sengelaub, 1994; Goldstein et al., 1996; Hays et al., 1996; Hebbeler and Sengelaub, 2003), due to dendritic interactions (Goldstein et al., 1993), or after NMDA receptor blockade (Hebbeler et al., 2002).

To assess potential redistributions of dendrites across treatment groups, for each animal the composite dendritic arbor created in the length analysis was divided using a set of axes radially oriented around the central canal, dividing the spinal cord into 12 bins of 30° each. The

portion of each animal's dendritic arbor per labeled motoneuron contained within each location was then determined.

Dendritic Extent. The comparability of BHRP labeling across groups was assessed by quantifying both the rostrocaudal and radial dendritic extent of SNB arbors. The rostrocaudal extent of the dendritic arbor was determined by recording the distance between the first and last section in which labeled SNB dendrites were present for each animal. In the transverse plane, for each animal the maximal radial extent of the dendritic arbor for each section throughout the rostrocaudal extent of the SNB dendritic field was measured using the same set of axes and 30° bins used for the dendritic distribution analysis. For each bin, the distance between the central canal and the most distal BHRP-filled process was measured.

Statistical analysis consisted of analyses of variance (one- or two-way with repeated measures) followed by appropriate planned comparisons (Fisher's Protected LSD) as described below. Digital light micrographs were obtained using an MDS 290 digital camera system (Eastman Kodak Company, Rochester, NY). Brightness and contrast of these images were adjusted in Adobe PhotoShop (Adobe Systems, Inc., San Jose, CA).

RESULTS

BC/LA Muscle Weight. BC/LA muscle weights did not differ across groups [$F(3,16) = 1.87$, n.s.; intact control males = 0.091 ± 0.01 g, castrates = 0.094 ± 0.01 g, castrates treated with estradiol implants from P7-P21 = 0.073 ± 0.02 g, castrates treated with estradiol implants from P7-P28 = 0.077 ± 0.02 g].

Morphometry. Injections of BHRP into the left BC successfully labeled ipsilateral SNB motoneurons in all animals in a manner consistent with previous studies (Kurz et al., 1986; Goldstein and Sengelaub, 1990; Goldstein and Sengelaub, 1994; Burke et al., 1997; Burke et al., 1999). SNB motoneurons displayed their characteristic multipolar morphologies, with dendritic arbors projecting ventrolaterally, dorsomedially, and across the midline into the area of the contralateral SNB (Fig. 2.3). The number of BHRP-labeled SNB motoneurons (51.30 ± 4.64) did not differ across groups [$F(3,19) = 2.39$, n.s.]

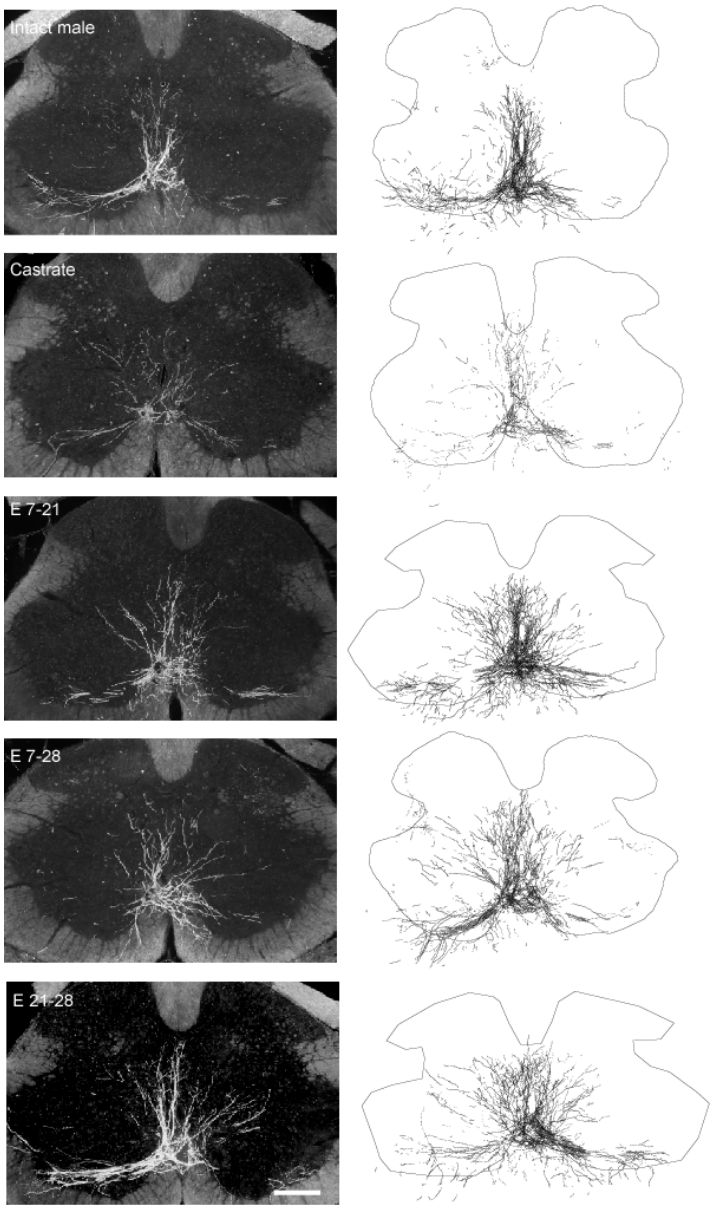

Figure 2.3. (Left) Darkfield digital micrographs of transverse sections through the lumbar spinal cord of an intact male (top), an untreated castrate (second from top), a castrate treated with an estradiol implant from P7-P21 (E7-21; second from bottom), and a castrate treated with estradiol from P7-P28 (E 7-28; bottom) after BHRP injection into the left BC muscle at P28. (Right) Computer-generated composites of BHRP-labeled somata and processes drawn at 320 μm intervals through the entire rostrocaudal extent of the SNB; these composites were selected as they are representative of their respective group average dendritic lengths. Scale bar = 250 μm.

Soma Area. The size of SNB somata at P28 differed significantly across groups [$F(3,19) = 6.63$, $p < 0.05$; Fig. 2.4]. The mean cross-sectional area of SNB somata in intact control males (837.77 ± 12.55 μm^2) was typical. The somata of untreated castrates (661.15 ± 21.11 μm^2, reduced 21%), or castrates treated with estradiol implants from P7-P21 (756.31 ± 101.4 μm^2, reduced 17%) or P7-P28 (647.26 ± 41.89 μm^2, reduced 23%) were all significantly smaller than those of intact control males (LSDs, $p < 0.05$).

Dendritic Length. The overall length of SNB dendrites significantly differed across groups [$F(3,19) = 17.02$, $p < 0.05$; Fig. 2.5]. SNB dendritic length in intact control males was typical (5963.02 ± 473.75 μm), reflecting the exuberant growth that occurs over the first four postnatal weeks. Dendritic lengths in castrates were approximately 67% shorter (1943.61 ± 604.17 μm) than those of intact control males at P28 (LSD, $p < 0.05$). Treatment of castrates with estradiol implants from P7-P21 or P7-P28 resulted in dendritic lengths (8237.88 ± 1146.67 μm, 8198.10 ± 417.01 μm, respectively) that were not different from those of intact control P28 males (LSDs, n.s.).

Dendritic Distribution. The SNB dendritic arbor of intact control males is radially organized but not uniformly distributed, with over 50% of the arbor concentrated ventrolaterally between 180 and 300° (Goldstein et al., 1993). The distribution of SNB dendrites showed an effect of group [repeated measures $F(3,209) = 17.39$, $p < 0.05$], as well as the typical significant effect of location [repeated measures $F(11,209) = 80.48$, $p < 0.05$; Fig. 2.6], and a group by location interaction [$F(33,209) = 6.971$, $p < 0.05$]. Castration not only reduced overall dendritic length, but it did so throughout the arbor, resulting in amounts of dendritic material per bin ranging from 26 to 42% of intact control male values [$F(3,19) = 17.02$, $p < 0.05$]. Castrates treated with estradiol implants from P7-P21 displayed a dendritic distribution that differed from that of intact control males [group by location interaction, $F(11,99) = 44.80$, $p < 0.05$]. This interaction was the result of an altered dendritic distribution in P7-P21 estradiol-treated castrates ventrolaterally between 210 and 240 degrees, where the amount of dendritic arbor was increased by 155% [$F(1,9) = 5.78$, $p < 0.05$]. Similarly, treating castrates with estradiol implants from P7-P28 resulted in an altered dendritic distribution, [group by location interaction, $F(11,88) = 2.60$, $p < 0.05$]. Again, this interaction was

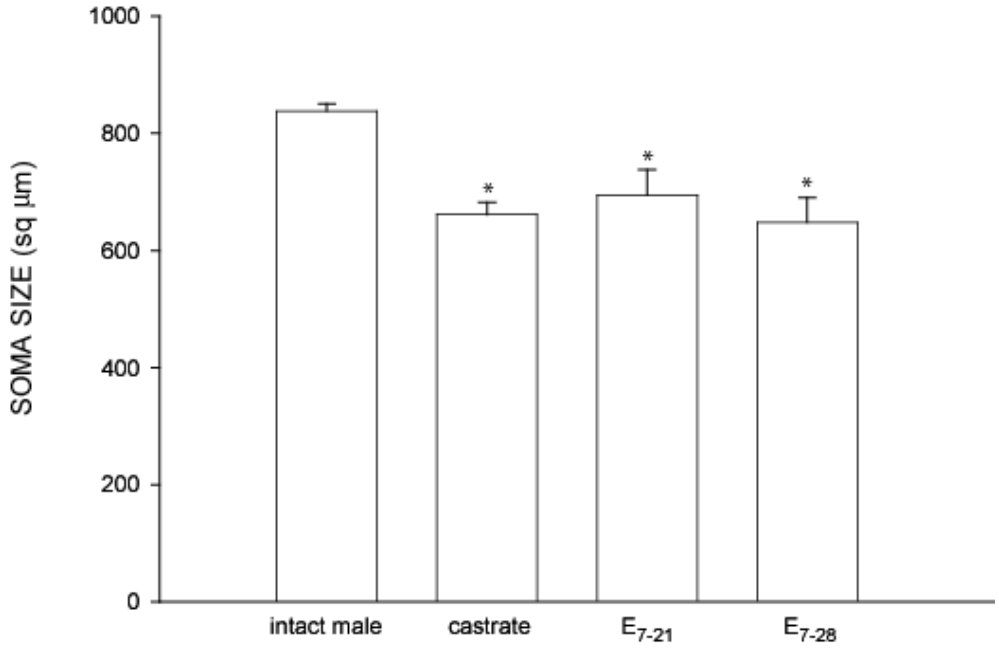

Figure 2.4. Soma areas of SNB motoneurons at P28 for intact males, untreated castrates (castrate), and castrates with estradiol implants from P7-P21 (E_{7-21}) or from P7-P28 (E_{7-28}). Bar heights represent means ± SEM for four to six animals per group. Asterisk denotes significantly different from intact control males.

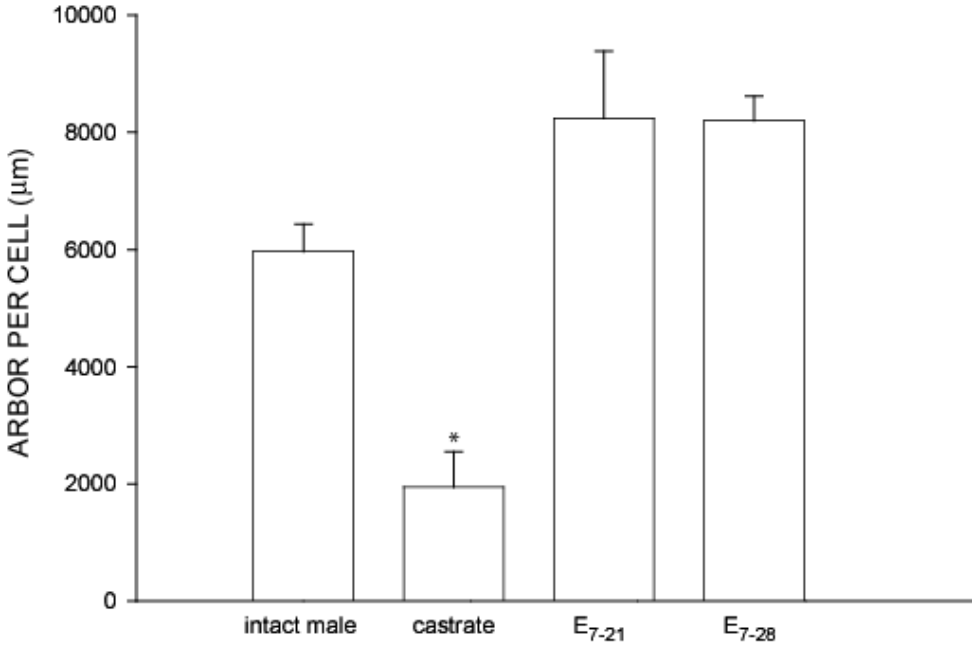

Figure 2.5. Dendritic lengths expressed as length of arbor per labeled SNB motoneuron at P28 for intact males, untreated castrates (castrate), and castrates with estradiol implants from P7-P21 ($E_{7\text{-}21}$) or from P7-P28 ($E_{7\text{-}28}$). Bar heights represent means ± SEM for four to six animals per group. Asterisk denotes significantly different from intact control males.

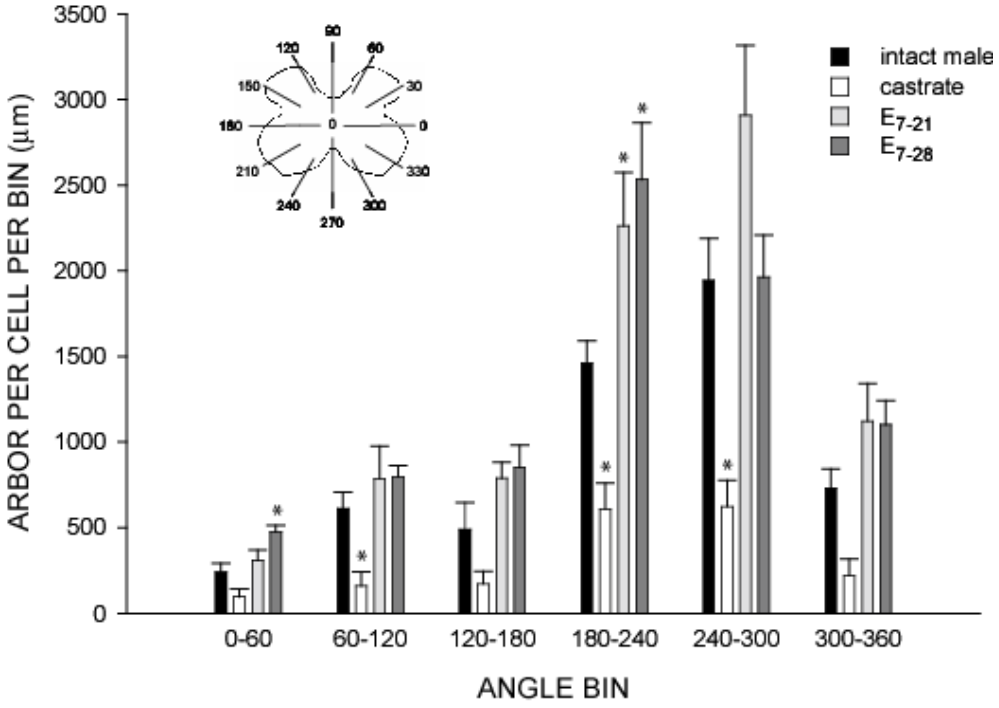

Figure 2.6. (Top) Schematic drawing of spinal gray matter divided into radial sectors for measure of SNB dendritic distribution. (Bottom) Length per radial bin of SNB dendrites at P28 for intact males, untreated castrates (castrate), and castrates with estradiol implants from P7-P21 ($E_{7\text{-}21}$) or from P7-P28 ($E_{7\text{-}28}$). For graphical purposes, dendritic length measures have been collapsed into six bins of 60° each. Bar heights represent means ± SEM for four to six animals per group. Asterisk denotes significantly different from intact control males.

the result of an altered dendritic distribution in P7-P28 estradiol-treated castrates ventrolaterally between 210-240 degrees [$F(1,8) = 10.78$, $p < 0.05$], where the amount of dendritic arbor was enhanced by 173%.

Dendritic Extent. The distance spanned by SNB dendrites in the rostrocaudal axis did not differ across treatment groups [$F(3,19) = 1.22$, n.s.; intact control males = 2432.00 ± 283.52 μm, castrates = 2377.14 ± 161.62 μm, castrates treated with estradiol from P7-P21 = 2693.33 ± 96.15 μm, castrates treated with estradiol from P7-P28 = 2752.00 ± 93.30 μm]. The radial extent of BHRP labeling showed an effect of group [$F(3,19) = 17.02$, $p < 0.05$; Fig. 2.7]. The effect of group was due to decreases in radial extent in castrates; radial extent did not differ between intact control males and estradiol-treated castrates [$F(2,13) = 0.17$, n.s.].

DISCUSSION

Immunolabeling for ERα in the BC muscle revealed the presence of ERα during development as well as in adulthood. The percentage of ERα-positive muscle fibers in the BC muscle did not change with age. In contrast, the density of ERα-positive extra-muscle fiber cells changed dramatically and was greatest during the early postnatal period, when estrogens act at the musculature to facilitate SNB dendritic growth, but absent in adulthood, when SNB motoneurons are insensitive to estrogens. The current results suggest that ERα located within these extra-muscle fiber cells could be the site of action for estrogenic support of SNB motoneuron dendrite growth during the first four weeks of postnatal development in male rats.

ERα Immunolabeling

Binding of estrogens by the BC/LA muscle has been previously reported (Dube et al., 1976), so the presence of ERs in this muscle was expected. However, our results describe a specific isoform of the receptor—ERα—and its expression in this tissue, and further demonstrate that the BC's ability to bind estrogens is present both throughout development and in adulthood. Interestingly, ERα immunostaining did not appear to be restricted to the nuclei of muscle fibers. This is consistent with previous reports of the localization of ERα in skeletal muscle, indicating the

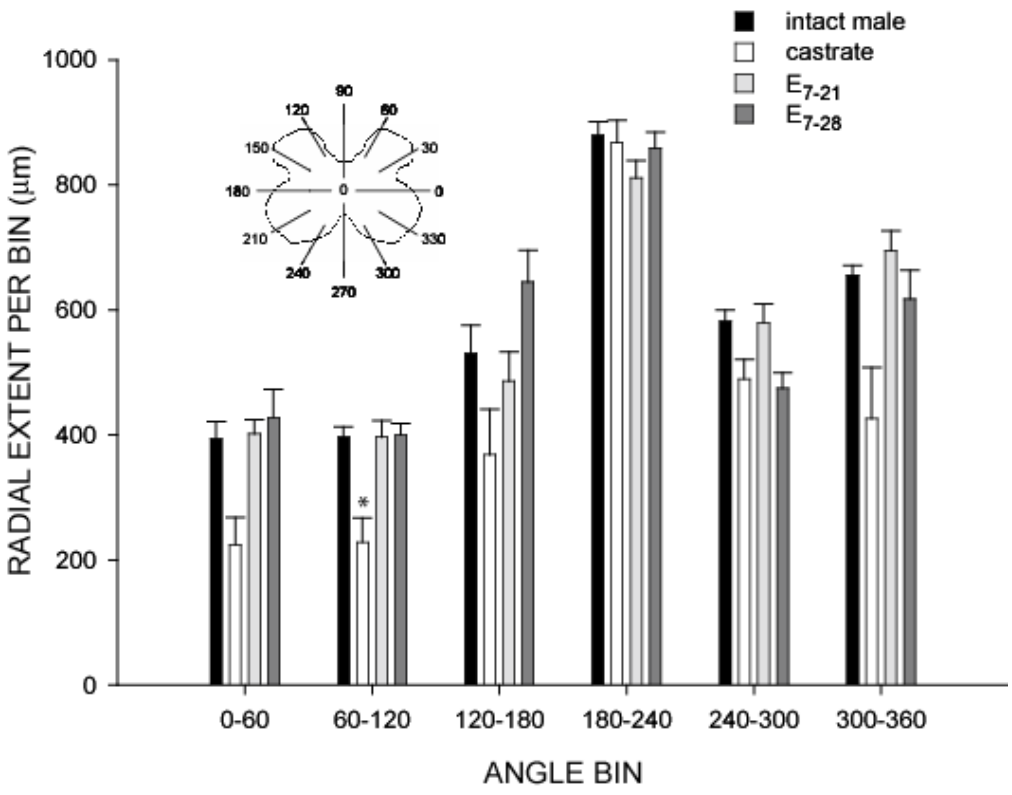

Figure 2.7. (Top) Schematic drawing of spinal gray matter divided into radial sectors for measure of SNB radial extent. (Bottom) Radial extents of SNB dendrites at P28 for intact males, untreated castrates (castrate), and castrates with estradiol implants from P7-P21 ($E_{7\text{-}21}$) or from P7-P28 ($E_{7\text{-}28}$). For graphical purposes, dendritic extent measures have been collapsed into six bins of 60° each. Bar heights represent means ± SEM for four to six animals per group.

presence of ERα in cytosol, and in association with particular subcellular fractions (e.g., mitochondrial; Milanesi et al., 2008).

Our results demonstrate that the percentage of ERα-positive muscle fibers did not differ across development or in adulthood. Because BC muscle fiber number is stable across the time points used in this study (Tobin and Joubert, 1991), measurements of ERα-positive muscle fibers are not biased by an alteration in total muscle fiber number. Furthermore, since there is a change in estrogen's effects on dendrites from development to adulthood, but the proportions of ERα-positive muscle fibers do not change during these time periods, it is unlikely that ERα expression within muscle fibers mediates these transient estrogenic effects on SNB dendrites. It is possible that the other predominant ER isoform—ERβ—could mediate the estrogenic effects on SNB dendrites via the BC. Therefore, not observing a change in ERα expression in muscle fibers does not rule out a role of muscle fibers in the estrogenic support of motoneuron dendrite growth during the early postnatal period. However, it is also possible that ERβ is not expressed in BC muscle, because there is great variability in the differential expression of ERα and ERβ depending on the target tissue and many other factors (Banie et al., 2008).

Although ERα expression in muscle fibers did not change across ages, the density of ERα-positive extra-muscle fiber cells was significantly different during early postnatal development compared to adult animals. We found that the density of ERα-positive extra-muscle fiber cells was significantly higher in P7 and P14 animals compared to adults. These results demonstrate that ERα is present in BC extra-muscle fiber cells during the time when estrogens act at this muscle to promote SNB dendritic growth. The density of ERα-positive extra-muscle fiber cells is substantially lower or zero at later ages, when SNB motoneurons are insensitive to estrogens. Since the expression of ERα in extra-muscle fiber cells coincides with the critical period for estrogen action at the muscle on SNB dendritic growth, this suggests that these cells could be the site of action for estrogen-mediated SNB dendritic growth during the early postnatal period.

Previous work has demonstrated that the robust growth of SNB dendrites during the first four postnatal weeks of life is steroid-dependent (Goldstein et al., 1990). These studies all used

treatment periods lasting through P28. Based on the changes in density of ERα-positive BC extra-muscle fiber cells we hypothesized that the critical period for estradiol exposure in supporting dendritic growth ended earlier than P28. Our results demonstrate that the period of high density of ERα-positive BC extra-muscle fiber cells may define the critical period for estrogen-dependent SNB dendrite growth during the early postnatal period: treating castrates with estradiol implants at the BC muscle when the density of ERα-positive extra-muscle fiber cells is high fully supports SNB dendrite growth, resulting in dendrite lengths that are not different from those of P28 intact control males or animals treated from P7-P28 with estradiol implants at the BC.

Target Musculature

BC/LA muscle development is androgen-dependent (Čihák et al., 1970; Tobin and Joubert, 1991). In males, the number of myotubes, mononucleate cells, and muscle fibers in the BC/LA increases during the first week of life, and exposure to androgens during the first week of life is sufficient to support BC/LA growth through P28 (Hebbeler and Sengelaub, 2003; Tobin and Joubert, 1991; present study). Furthermore, because BC/LA weights did not differ, any difference in SNB morphology could not be due to differences in muscle size.

Motoneuron Morphology

Somata. In normal males most SNB somal growth occurs during the first four postnatal weeks, and this increase in soma size is androgen-dependent (Breedlove and Arnold, 1983; Breedlove and Arnold, 1983; Goldstein and Sengelaub, 1990; Goldstein and Sengelaub, 1994). In the present study, SNB somata in castrates were smaller than those of intact control males, consistent with previous findings demonstrating the androgen-dependence of SNB soma growth. Treatment of castrates with estradiol (from P7-P21 or P7-P28) did not support SNB somal growth during the first four postnatal weeks, consistent with previous work demonstrating that estradiol fails to masculinize SNB soma size (Breedlove et al., 1982; Goldstein and Sengelaub, 1994; Burke et al., 1997; Hebbeler et al., 2002; Nowacek and Sengelaub, 2006) and blocking synthesis of estrogens with aromatase inhibition does not interfere with the development of masculine soma size (Burke et al., 1999).

Dendrites. Our results demonstrate that castration results in significantly reduced SNB dendrite lengths, replicating previous findings (Goldstein et al., 1990; Goldstein and Sengelaub, 1994; Nowacek and Sengelaub, 2006). Furthermore, these results confirm the neuromuscular periphery as the site of action for estrogen-dependent SNB dendrite growth during the early postnatal period (Nowacek and Sengelaub, 2006). ERs are found in non-muscle fiber cells, including terminal Schwann cells (Jung-Testas et al., 1993). In the current study, we demonstrated that ERα expression in a BC extra-muscle fiber cell type coincides with the early postnatal period during which estradiol acts at the target musculature to promote SNB dendrite growth. Together, these data suggested that ERα expression in the BC muscle could be the site of action for mediating the critical period for estrogen-dependent SNB dendrite growth during the early postnatal period. In the present study, we restricted estradiol treatment at the BC muscle to the period when the density of ERα-positive extra-muscle fiber cells was highest (P7-P21). Our results support the hypothesis that the period of estrogen-dependent SNB dendrite growth is potentially defined by the presence of ERα in extra-muscle fiber cells in the BC, as animals treated with estradiol implants from P7-P21 had dendritic arbors that were not different than intact control animals or castrates treated with estradiol implants from P7-P28.

Previous work has demonstrated the importance of the target musculature in the regulation of estradiol-dependent SNB dendrite growth. Whereas estrogens are important during early postnatal development for SNB dendritic growth, SNB motoneurons do not accumulate estradiol during development (Taylor et al., 1995) or in adulthood (Breedlove and Arnold, 1980; Breedlove and Arnold, 1983), suggesting that these hormones exert their effect of SNB dendritic growth indirectly. Neither primary sensory nor supraspinal afferents appear to be involved in SNB dendritic growth (Goldstein et al., 1996; Hays et al., 1996; Hebbeler and Sengelaub, 2003), and it has been suggested that the growth and differentiation of dendrites may be initiated and shaped by contact with the target tissues (Sumner and Watson, 1971; Yawo, 1987; Voyvodic, 1989). Furthermore, blocking androgen receptors at the BC muscle in adulthood results in a reduction of SNB dendritic lengths, demonstrating that hormone receptors in target tissues are critical for the target-mediated hormonal regulation of SNB morphology (Rand and Breedlove, 1995). Our data

provide further evidence that estradiol-dependent SNB dendrite growth is regulated at the target musculature, and indicate that hormone receptor expression in BC muscles mediates this target-dependent dendrogenesis.

Similarly, our results indicate that hormone receptor expression in the target musculature can regulate motoneuron dendrite morphology. Recent work has demonstrated that there is a causal relationship between androgen receptor expression in the target musculature and degree of androgen sensitivity exhibited by the innervating motoneurons (Huguenard et al., 2011). By manipulating the expression of androgen receptors exclusively in the target musculature, androgen sensitivity was induced in a population of normally androgen-insensitive motoneuron dendrites, demonstrating that androgen sensitivity is directly conferred by receptor expression in the target musculature (Huguenard et al., 2011). Along with our findings, these results further implicate the importance of receptor expression in the target muscles for hormonal regulation of motoneuron morphology.

When examining the differences in dendritic distribution across groups, we found that treatment of castrates with estradiol at the BC muscle (either from P7-P21 or P7-P28) resulted in significantly longer SNB dendrites in the gray matter ventrolateral to the central canal compared to intact control animals. This area of SNB dendritic arbor where estrogen treatment promotes enhanced dendritic growth compared to intact control males has previously been identified as an important source of excitatory drive to the SNB (Tanaka and Arnold, 1993), and shows specific reductions in dendritic length following spinal transection early in development (Hebbeler and Sengelaub, 2003). Future research should determine why estradiol treatment at the BC muscle in castrates drives SNB dendritic development in this area of the arbor that is specialized for receiving excitatory inputs.

Comparability of BHRP labeling

Previous studies have demonstrated that neither axonal transport of BHRP (Leslie et al., 1991) nor dendritic transport as demonstrated by the rostrocaudal or mediolateral extent of dendritic labeling (Kurz et al., 1990; Goldstein and Sengelaub, 1994) is affected by hormone levels. In the present study, the possibility that hormone manipulations could affect retrograde

transport is an important consideration, as such artifact could potentially result in apparent alterations in dendritic morphology. No difference in rostrocaudal extents of SNB dendrites were observed, but radial extents in untreated castrates were smaller than those of intact control males. This result most likely reflects the attenuated growth of SNB dendrites rather than a transport artifact, which (because rostrocaudal extent was not affected) would necessarily have had to occur selectively in the transverse plane. In contrast, no differences in either rostrocaudal or radial extents of dendrites were observed between intact control males and estradiol-treated castrates, indicating that the ability of SNB dendrites to transport BHRP out to the most distal, highest order branches was not affected in these groups. Thus, we believe the dendritic labeling across groups was comparable, allowing direct comparisons of dendritic length and distribution across groups.

Mechanisms of target-mediated dendritic growth

Specific features of the SNB system, including perineal muscle development and motoneuron morphology, are regulated by testosterone action at androgen receptors during a perinatal critical period (Freeman et al., 1996). To test the hypothesis that androgen receptors in BC/LA muscle fibers are directly responsible for testosterone-mediated masculinization of the SNB system, Niel et al. (2009) created a transgenic animal that expressed androgen receptors only in muscle fibers, but not other cell types. Despite the expression of androgen receptors, SNB motoneurons and the LA muscle exhibited the female phenotype in these animals, demonstrating that androgen receptor expression within muscle fibers was not sufficient to mediate sexual differentiation in this system. These authors suggested, as we do, that it is the expression of hormone receptors in a non-muscle fiber cell type in the BC/LA that is responsible for hormone effects on the SNB neuromuscular system (Niel et al., 2009).

These extra-muscle fiber cells could be fibroblasts, adipocytes, endothelial cells, epithelial cells, or Schwann cells, and it could be a change in ER in these cells or a change in the amount of these cells themselves, that mediates the change in SNB estrogen sensitivity from the early postnatal period to adulthood. While it is unclear how estradiol action at extra-muscle fiber ERα promotes SNB dendrite growth, a possible mechanism of action could be through the

regulation of growth factors. For example, in adulthood, androgens regulate brain-derived neurotrophic factor (BDNF) expression in the target musculature, and BDNF can reduce SNB dendritic length (Verhovshek et al., 2010a; Verhovshek and Sengelaub, 2010). Similarly, the ERα-positive extra-muscle fiber cells present postnatally could be supporting dendritic growth by suppressing BDNF expression during the early postnatal period. Future studies are needed to determine the identity of the extra-muscle fiber cells and their role during development (see Chapter 5).

In conclusion, the current data suggest that changes in ERα expression in the BC could define the critical period in which estrogens act at the target musculature to regulate SNB dendritic growth during the early postnatal period. Why this expression in extra-muscle fiber cells is limited to the postnatal period, and the mechanism by which it supports SNB dendrite growth remains to be determined.

CHAPTER 3: Rudolph, L.M. & Sengelaub, D.R. (2013). Castration-induced upregulation of muscle ERα supports estrogen sensitivity of motoneuron dendrites in a sexually dimorphic neuromuscular system. *Developmental Neurobiology.* 73(12): 921-935.

BACKGROUND

In Chapter 2, I demonstrated that ERα expression in BC extra-muscle fiber cells coincides with the period for estrogen-dependent dendrite growth in the SNB, and potentially mediates the estrogen sensitivity of SNB morphology (Rudolph and Sengelaub, 2013a). ERα expression in extra-muscle fiber cells of the SNB target muscle could regulate the estrogen sensitivity of SNB dendrites: extra-muscle fiber ERα is high during the first few weeks of life, declining through P21, and is reduced or absent in adulthood, when SNB dendrites are unresponsive to estrogens. Treatment of castrated males with estradiol restricted to the period of elevated ERα expression in BC extra-muscle fiber cells (P7-P21) results in full masculinization of SNB dendritic morphology at P28 (Rudolph and Sengelaub, 2013a). Therefore, it appears that ERα expression in extra-muscle fiber cells is critical for estrogen-regulated masculinization of SNB motoneuron morphology during development. Similarly, the masculinization of SNB motoneuron number is thought to be mediated by hormone action at non-muscle fiber cells in the target musculature (Niel et al., 2009). These data indicate a clear role for gonadal hormone receptor expression in extra-muscle fiber cells of the SNB target musculature in the developmental masculinization of SNB motoneurons.

The developmental expression patterns of ERα in BC muscle coincide with the transient period of estrogen sensitivity of SNB dendrites (Rudolph and Sengelaub, 2013a). However, it is unclear why levels of ERα in extra-muscle fiber cells decrease during the postnatal period. It is possible that ERα in BC extra-muscle fiber cells is downregulated by gonadal hormones. In female rat dorsal root ganglia, ER mRNA levels are low during proestrus (when circulating estrogens are elevated; Sohrabji et al., 1994). Levels of ER mRNA are high after removal of estrogens by ovariectomy, and estrogen treatment of ovariectomized animals reduces ER mRNA to levels of those found in proestrus animals (Sohrabji et al., 1994). Similarly, ovariectomy-induced increases in ERα mRNA and protein occur in skeletal muscles of female mice (Baltgavis et al., 2010). ERα mRNA in soleus and extensor digitorum longus muscles and ERα protein in tibialis anterior muscle increase significantly after castration, and either acute (48 hour) or chronic (21 day) estradiol treatment in castrates returns muscle ERα levels to those of intact animals

(Baltgavis et al., 2010). Together, these data suggest that developmental downregulation of extra-muscle fiber cell ERα in the SNB target muscle is potentially caused by the presence of gonadal hormones. We hypothesized that castration would result in an attenuation of the developmental decrease in ERα in the SNB target musculature. In Experiment 1, we castrated male rats on P7 and measured ERα immunolabeling in the BC muscle at P21 and compared this immunolabeling to that of gonadally intact P21 males.

Experiment 1

MATERIALS AND METHODS

Animals. Untimed pregnant rat dams (Sprague-Dawley, Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle, with unlimited access to food and water. When pregnant females gave birth (day of birth = P1), pups were sexed and litters were culled to eight pups when necessary, retaining males preferentially. One group of males remained gonadally intact (n = 6) and another group was bilaterally castrated under isoflurane anesthesia at P7 (n = 6). All procedures were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Bloomington Institutional Animal Care and Use Committee.

Immunohistochemistry. At P21 animals were weighed, given an overdose of urethane (0.5 ml/100 g of body weight), and perfused transcardially with 0.9% saline followed by cold 4% paraformaldehyde in 0.1M phosphate buffer (pH = 7.4). The BC/LA muscles were removed and postfixed in the same fixative for 24 h and transferred to 30% sucrose in 0.1M phosphate buffer for a minimum of 24 h of cryoprotection. The BC/LA was cut horizontally into 12 µm sections on a cryostat at -16°C. Sections were thaw-mounted onto gelatin-coated slides, and stored at -16°C. Immunohistochemistry proceeded according to previously used methods (Rudolph and Sengelaub, 2013a; Chapter 2).

Microscopic Analysis. Because all of our previous developmental work on SNB motoneuron dendritic growth, hormone sensitivity, critical period effects, and the characterization of developmental changes in ERα was done in BC-projecting motoneurons, the current analysis

was limited to the BC muscle, as it was in Chapter 2. Sections were first viewed under epifluorescent illumination to visualize basal lamina staining. The BC is a relatively complex muscle, with muscle fibers traveling in several different directions, resulting in fields containing both cross- and longitudinally-sectioned muscle fibers (Fargo et al., 2003); fields containing large numbers of basal lamina-stained muscle fibers in cross-section were selected for analysis. Using Stereo Investigator (MBF Bioscience, Inc., Williston, VT), muscle fibers were sampled systematically at 2200X (final magnification on display), by superimposing a grid on the tissue image (e.g., 75 μm x 75 μm square frame) and sampling all fibers within the frame; only fields containing a minimum of 23 muscle fibers were examined.

To quantify ERα immunostaining, muscle fibers were visualized under FITC fluorescence and images were captured for subsequent analysis. Areas that appeared to be labeled relative to background (see below) were identified as either in-fiber or extra-fiber depending on the location of ERα label relative to the basal lamina stain. One field per section and 1-2 sections per animal were analyzed; overall, an average 76.5 muscle fibers per animal were traced. For each analyzed tissue section, the mean and standard deviation of muscle fiber FITC luminance values were calculated and a minimum labeling criterion of the mean plus two standard deviations was established for each section. Cells that exceeded this criterion were identified as ERα-positive and were categorized as either ERα-positive muscle fibers (if surrounded by a basal lamina) or ERα-positive extra-muscle fiber cells. We sampled equivalent numbers of muscle fibers across groups and expressed ERα-positive muscle fibers as a percentage of the sample. ERα-positive extra-muscle fiber cells were identified only on the basis of ERα labeling, and their numbers were expressed as a density per unit area or as a ratio relative to the number of muscle fibers sampled. The percentage of ERα-positive muscle fibers and density of ERα-positive extra-muscle fiber cells per section were analyzed using one-way ANOVAs with Fisher's LSD post-hoc tests. Differences were considered significant if $p < 0.05$.

RESULTS

As reported previously (Rudolph and Sengelaub, 2013a), ERα immunolabeling was present in both muscle fibers (label contained within the basal lamina stain) and extra-muscle fiber cells (label outside basal lamina stain; Fig. 3.1). The average number of muscle fibers analyzed per animal did not differ between groups [$F(1,10) = 0.25$, n.s.]. For each animal, the number of labeled muscle fibers was expressed as a percentage of total muscle fibers sampled. The percentage of ERα-positive muscle fibers did not differ between normal (3.15 ± 1.12%) and castrated males [2.65 ± 1.02%; $F(1,10) = 0.11$, n.s.; Fig. 3.2].

There was a significant main effect of castration on the density of ERα-positive extra-muscle fiber cells. ERα expression in extra-muscle fiber cells was significantly greater in castrated animals (740.74 ± 148.15 per mm^2) compared to that of normal animals (109.26 ± 46.83 per mm^2); [$F(1,10) = 16.52$, $p < 0.05$; Fig. 3.2]. Similarly, the number of ERα-positive extra-muscle fiber cells relative to the number of muscle fibers sampled was significantly increased in castrated animals (8.67 ± 1.25 per 100 muscle fibers) compared to that of normal animals (1.47 ± 0.60 per 100 muscle fibers); [$F(1,10) = 27.17$, $p < 0.01$; Fig. 3.2]. Changes in androgens during the postnatal period examined in this experiment have no effect on muscle weight (Hebbeler and Sengelaub, 2003; Experiment 2, current chapter) or fiber number in the SNB target musculature (Tobin and Joubert, 1991). Thus the increase in ERα-positive extra-muscle fiber cells as a function of area or relative to the number of muscle fibers sampled is not likely a result of changes in BC/LA size or fiber number.

Experiment 2

In Experiment 1, we found that the density of ERα-positive BC extra-muscle fiber cells at P21 was significantly greater in animals castrated at P7 compared with normal males. Based on these results, and earlier data demonstrating that extra-muscle fiber ERα expression coincides with the critical period for estrogen-dependent SNB dendrite growth, we hypothesized that the elevated ERα expression in BC extra-muscle fiber cells in castrated animals would result in heightened estrogen sensitivity of SNB dendrites. To test this hypothesis, in Experiment 2 we

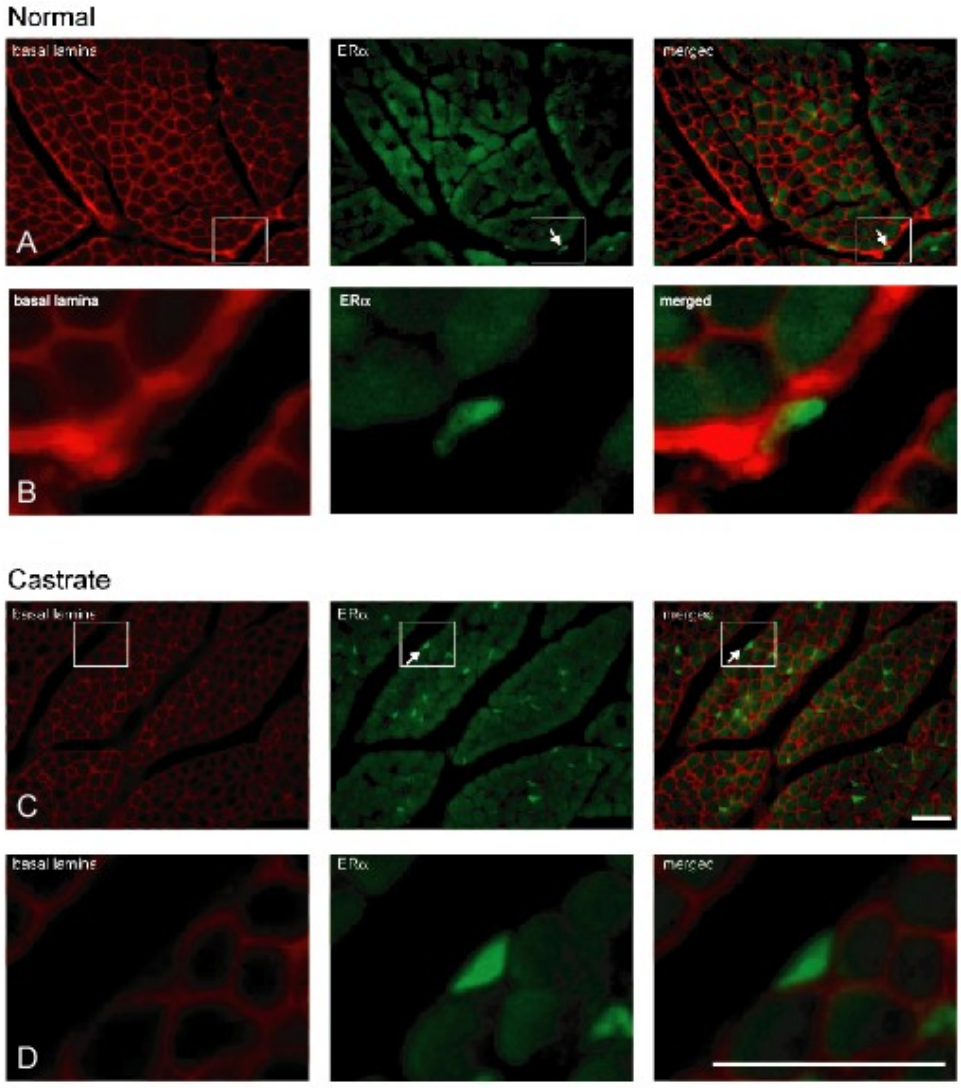

Figure 3.1. Digital light micrographs of cross-sections of BC muscle in normal (A, B) and castrated (C, D) male rats at P21; higher magnification views (B, D) of indicated areas. Each row shows the same tissue section viewed under different epifluorescent illumination, demonstrating immunolabeling for basal lamina only (red; left), ERα only (green; middle), and the merged image showing combined basal lamina and ERα immunolabeling (right). Arrows indicate ER□-positive extra-muscle fiber cells; note the absence of basal lamina staining of the indicated extra-muscle fiber cells in rows B, D. Scale bars = 25 μm.

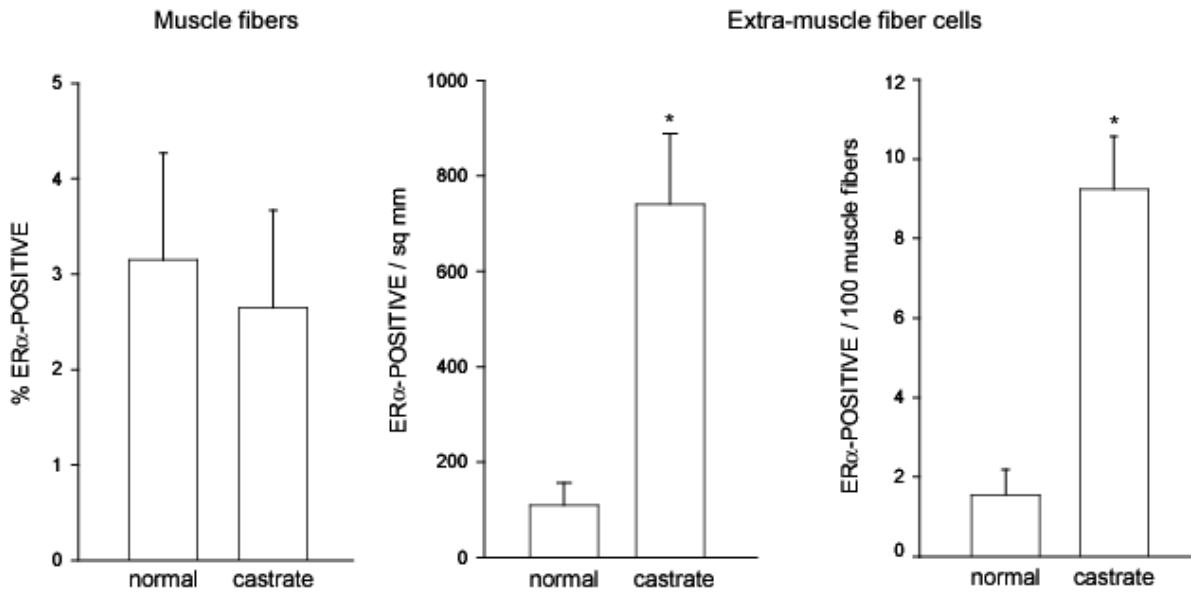

Figure 3.2. The percentage of ERα-positive muscle fibers (left), density of ERα-positive extra-muscle fiber cells (middle), and ratio of ERα-positive extra-muscle fiber cells to muscle fibers in the BC muscle at P21 in normal males and males castrated at P7. Bar heights represent means ± SEM for six animals per group. * denotes significantly different from normal males.

assessed SNB dendritic morphology at P28 in normal males, untreated castrates, castrates treated with estradiol implants from P21 to P28, and normal males treated with estradiol implants from P21 to P28.

MATERIALS AND METHODS

Animals. Untimed pregnant rat dams (Sprague-Dawley; Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle, with unlimited access to food and water. Litters were culled to eight pups when necessary, retaining males preferentially. Males were bilaterally castrated under isoflurane anesthesia at P7. One group of castrates had estradiol implants placed at the left BC muscle at P21, remaining until P28 (n = 7); another group of castrates remained untreated (n = 7). Age-matched normal males were also included, one group was left untreated (n = 5) and another group received estradiol implants at the left BC muscle from P21 to P28 (n = 5; overall n = 24). All procedures were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Bloomington Institutional Animal Care and Use Committee.

Implants. To restrict hormone treatment to the SNB target musculature, we used our previous method (Nowacek and Sengelaub, 2006; Rudolph and Sengelaub, 2013a; Chapter 2) to deliver estradiol locally to the BC muscle.

Histochemistry. Horseradish peroxidase conjugated to the cholera toxin β subunit (BHRP; List Biological, Campbell, CA) was used to retrogradely label SNB motoneurons innervating the BC muscle, as previously described (Chapter 2).

Motoneuron Somata. The number of BHRP-filled motoneurons was assessed in all sections through the entire rostrocaudal extent of the SNB for all animals, as previously described (Chapter 2). The cross-sectional soma area of BHRP-labeled motoneurons was measured in an average of 22.63 motoneurons for each animal.

Dendritic Length. For each animal, dendritic lengths in a single representative set of alternate sections were measured under darkfield illumination. Potential redistributions of dendrites as well

as the comparability of BHRP labeling across treatment groups were assessed using previously described methods (Rudolph and Sengelaub, 2013a; Chapter 2).

RESULTS

BC/LA Muscle Weight. Body weights differed across groups [$F(3,17) = 3.89$, $p < 0.05$], being largest in normal males and castrated males (LSD, n.s.) and smaller in estradiol-treated animals (LSD, n.s.; Table 3.1). BC/LA muscle weight showed an identical pattern of differences [$F(3,17) = 19.47$, $p < 0.01$; Table 3.1]. Differences in muscle weight remained after correcting for differences in body weight [raw muscle weight / body weight) x 100]: BC/LA muscle weights showed a significant effect of group [$F(3,17) = 22.94$, $p < 0.01$; normal males = 0.100 ± 0.002 g, castrates = 0.094 ± 0.004 g, castrates treated with estradiol implants from P21-P28 = 0.072 ± 0.002 g, normal males treated with estradiol from P21-P28 = 0.077 ± 0.004 g]. The corrected weights of BC/LA muscles from animals treated with estradiol implants were significantly lower than those of normal and castrated males (LSDs, $p < 0.01$).

Morphometry. Injections of BHRP into the left BC successfully labeled ipsilateral SNB motoneurons in all animals in a manner consistent with previous studies (Kurz et al., 1986; Goldstein and Sengelaub, 1990; Goldstein and Sengelaub, 1994; Burke et al., 1997; Burke et al., 1999). SNB motoneurons displayed their characteristic multipolar morphologies, with dendritic arbors projecting ventrolaterally, dorsomedially, and across the midline into the area of the contralateral SNB (Fig. 3.3). The number of BHRP-labeled SNB motoneurons (61.17 ± 5.59) did not differ across groups [$F(3,20) = 0.02$, n.s.; Table 3.1].

Soma Area. The size of SNB somata at P28 differed significantly across groups [$F(3,20) = 3.90$, $p < 0.05$; Fig. 3.4]. The mean cross-sectional area of SNB somata in normal males (749.30 ± 23.78 μm^2) was typical. The somata of untreated castrates (661.15 ± 21.11 μm^2, reduced 12%), or castrates treated with estradiol implants from P21-P28 (638.18 ± 13.77 μm^2, reduced 15%) were both significantly smaller than those of normal males (LSDs, $p < 0.05$). The areas of SNB somata of normal males treated with estradiol from P21-P28 (702.92 ± 40.43 μm^2) were not different than those of untreated normal males (LSD; n.s.).

Table 3.1. Raw body and muscle weights and number of BHRP-labeled motoneurons (Means ± SEM) in normal males, castrated males, castrated males treated with estradiol from P21-P28, and normal males treated with estradiol from P21-P28

	Normal	Castrate	Castrate + E_{21-28}	Normal +E_{21-28}
Body weight (g)	89.40 ± 5.86	100.25 ± 4.73	85.57 ± 2.07†	79.40 ± 4.18†
BC/LA weight (g)	0.091 ± 0.007	0.094 ± 0.005	0.061 ± 0.001*†	0.060 ± 0.002*†
BHRP-labeled motoneurons	60.00 ± 11.38	63.43 ± 7.58	60.57 ± 10.16	60.00 ± 19.8

*Significantly different from normal males

†Significantly different from castrated males

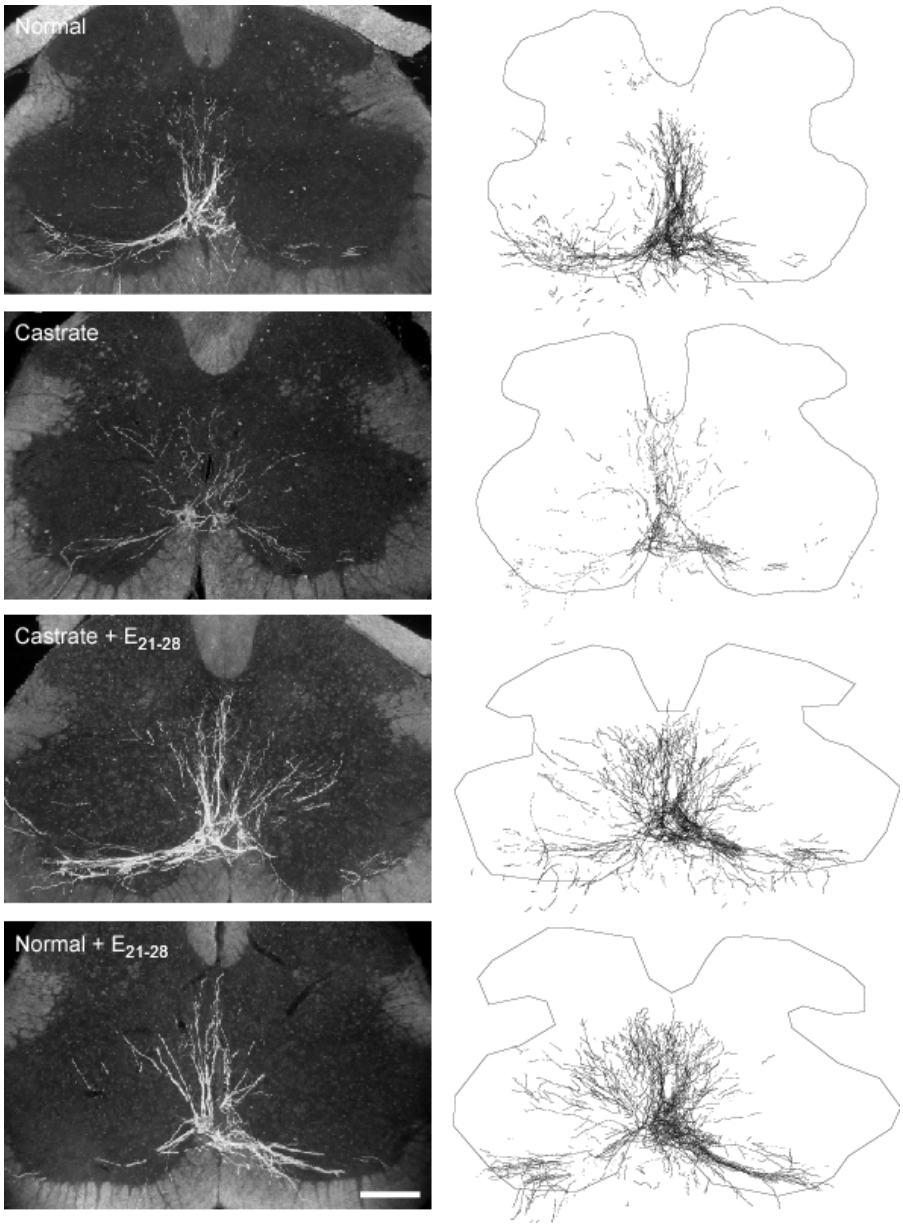

Figure 3.3. (Left) Darkfield digital micrographs of transverse sections through the lumbar spinal cord of a normal male (top), an untreated castrate (second from top), a castrate treated with an estradiol implant from P21-P28 (Castrate + $E_{21\text{-}28}$; second from bottom), and a normal male treated with an estradiol implant from P21-P28 (Normal + $E_{21\text{-}28}$; bottom) after BHRP injection into the left BC muscle at P28. (Right) Computer-generated composites of BHRP-labeled somata and processes drawn at 320 µm intervals through the entire rostrocaudal extent of the SNB; these composites were selected as they are representative of their respective group average dendritic lengths. Scale bar = 250 µm.

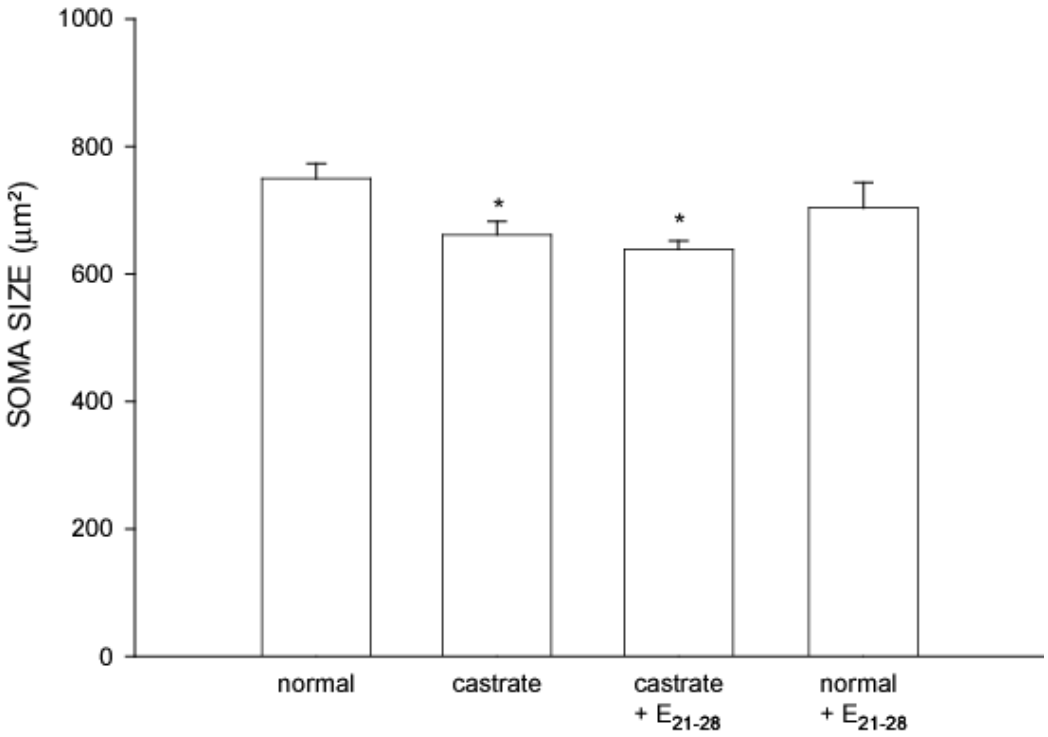

Figure 3.4. Soma areas of SNB motoneurons at P28 for normal males, untreated castrates (castrate), castrates treated with estradiol implants from P21-P28 (castrate + $E_{21\text{-}28}$), and normal males treated with estradiol implants from P21-P28 (normal + $E_{21\text{-}28}$). Bar heights represent means ± SEM for four to seven animals per group. * denotes significantly different from normal males.

Dendritic Length. The overall length of SNB dendrites significantly differed across groups [$F(3, 20) = 14.97$, $p < 0.01$; Fig. 3.5]. SNB dendritic length in normal males was typical (5963.02 ± 473.75 μm), reflecting the exuberant growth that occurs over the first four postnatal weeks. Dendritic lengths in castrates were approximately 67% shorter (1943.61 ± 604.17 μm) than those of normal males (LSD, $p < 0.05$). Castrates treated with estradiol from P21-P28 had SNB dendritic arbors (8978.28 ± 1150.46 μm) that were significantly greater than those of normal males and untreated castrates (LSD, $p < 0.05$). Dendritic lengths of normal males treated with estradiol from P21-P28 (4708.33 ± 420.29 μm) were no different from those of untreated normal males (LSD, n.s.).

Dendritic Distribution. The SNB dendritic arbor of normal males is radially organized but not uniformly distributed, with over 50% of the arbor concentrated ventrolaterally between 180 and 300° (Goldstein et al., 1993). The distribution of SNB dendrites showed an effect of group [repeated measures $F(3,209) = 13.03$, $p < 0.01$], as well as the typical significant effect of location [repeated measures $F(11,209) = 42.04$, $p < 0.01$; Fig. 6], and a group by location interaction [$F(33,209) = 3.12$, $p < 0.01$]. Castration not only reduced overall dendritic length, but it did so throughout the arbor, resulting in amounts of dendritic material per bin ranging from 26 to 42% of normal male values [$F(1,10) = 23.31$, $p < 0.05$]. Treatment of castrates with estradiol from P21-P28 not only prevented reductions in dendrite lengths [$F(1,11) = 35.40$, $p < 0.05$], but resulted in dendritic hypertrophy throughout the arbor [$F(1,9) = 8.30$, $p < 0.05$], with dendritic arbors of P21-P28 estradiol-treated castrates ranging from 152-234% of those of normal males. In normal males, SNB dendritic distribution was not affected by estradiol treatment from P21-P28 [$F(1,8) = 0.04$, n.s.].

Dendritic Extent. The distance spanned by SNB dendrites in the rostrocaudal axis showed a difference across groups [$F(3,20) = 3.61$, $p < 0.05$; normal males = 2720.00 ± 208.61 μm, castrates = 2377.14 ± 161.62 μm, castrated treated with estradiol from P21-P28 = 2971.43 ± 109.62 μm, normal males treated with estradiol from P21-P28 = 2976.00 ± 148.38 μm]. This effect of group was due to decreases in rostrocaudal extent of dendrites in castrates; rostrocaudal extent did not differ between normal males and estradiol treated animals [$F(2,14) = 0.87$, n.s.].

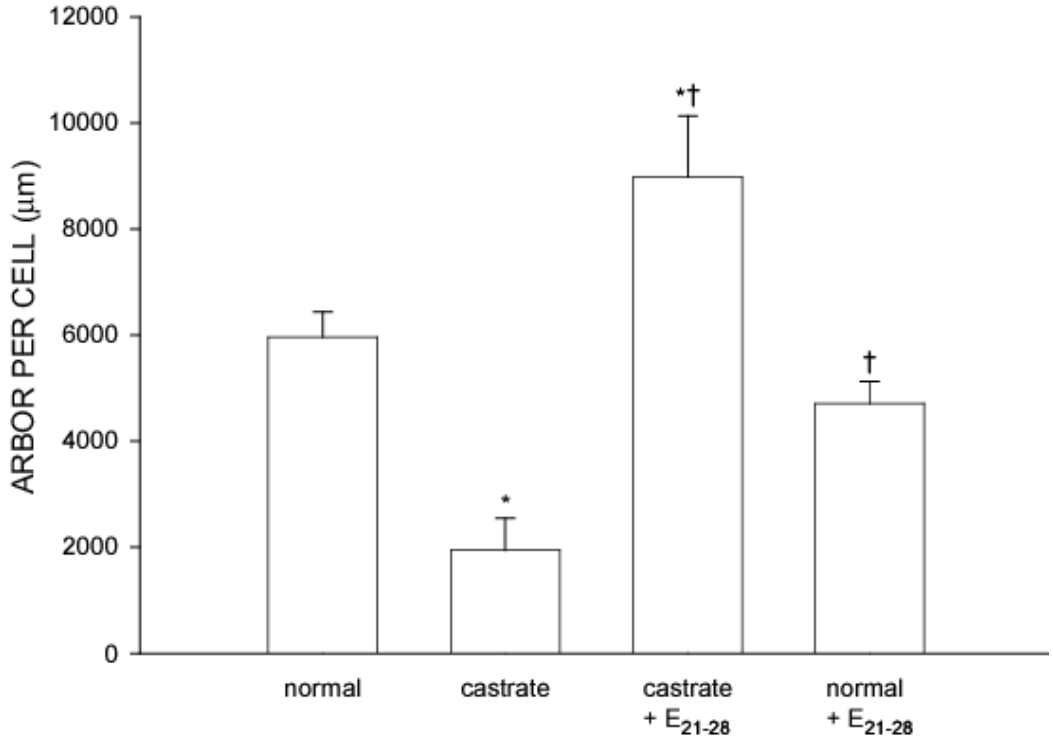

Figure 3.5. Dendritic lengths expressed as length of arbor per labeled SNB motoneuron at P28 for normal males, untreated castrates (castrate), castrates treated with estradiol implants from P21-P28 (castrate + $E_{21\text{-}28}$), and normal males treated with estradiol implants from P21-P28 (normal + $E_{21\text{-}28}$). Bar heights represent means ± SEM for four to seven animals per group. * denotes significantly different from normal males; † indicates significantly different from castrated males.

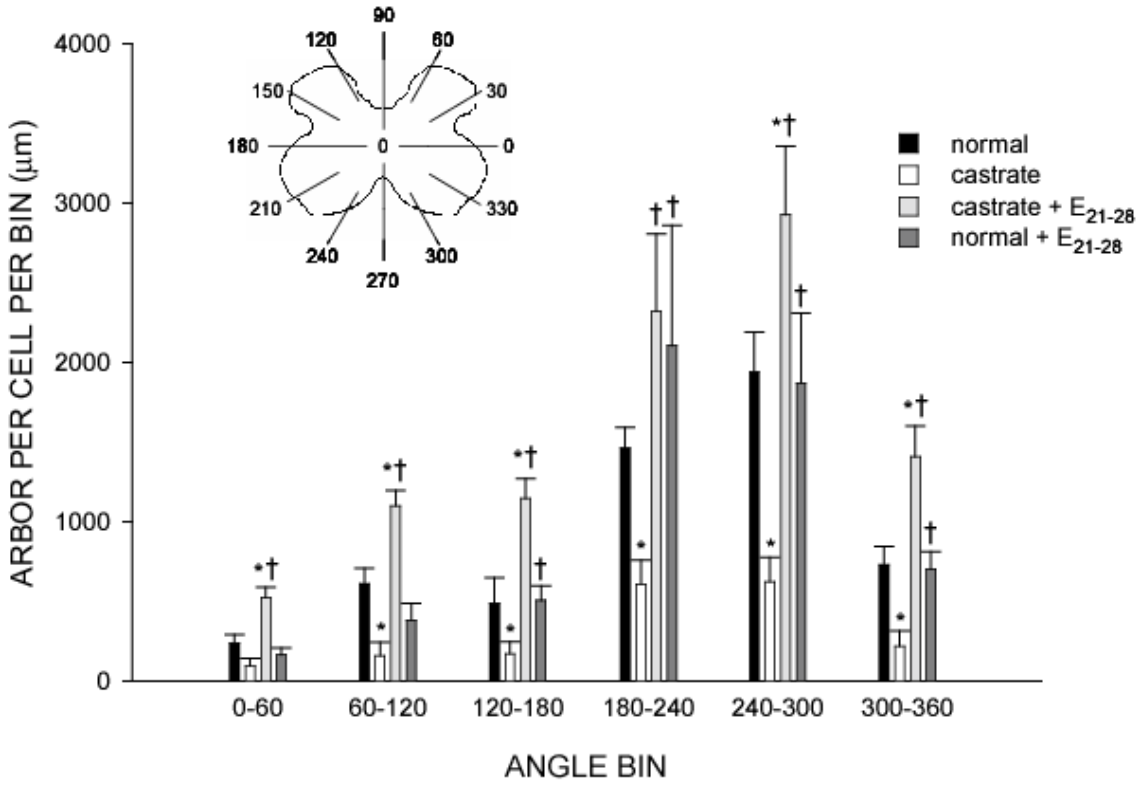

Figure 3.6. (Inset) Schematic drawing of spinal gray matter divided into radial sectors for measure of SNB dendritic distribution. Length per radial bin of SNB dendrites at P28 for normal males, untreated castrates (castrate), castrates treated with estradiol implants from P21-P28 (castrate + $E_{21\text{-}28}$), and normal males treated with estradiol from P21-P28 (normal + $E_{21\text{-}28}$). For graphical purposes, dendritic length measures have been collapsed into six bins of 60° each. Bar heights represent means ± SEM for four to seven animals per group. * denotes significantly different from normal males; † indicates significantly different from castrated males.

The radial extent of BHRP labeling showed an effect of group [$F(3,19) = 3.79$, $p < 0.05$]. The effect of group was due to decreases in radial extent in castrates; radial extent did not differ between normal males and estradiol-treated animals [$F(2,13) = 2.73$, n.s.].

DISCUSSION

Immunolabeling for ERα in the BC muscle at P21 in castrated and gonadally intact males revealed ERα expression in muscle fibers and extra-muscle fiber cells. While the percentage of ERα-positive muscle fibers was unaltered by castration, the density of extra-muscle fiber cells immunolabeled for ERα was significantly greater in castrates compared with gonadally intact males. The castration-induced increase in muscle ERα appears to have functional consequences for SNB morphology, as 7 days of estradiol treatment was sufficient not only to masculinize SNB dendrites at P28, but cause dendritic growth that exceeded that of normal males. These data demonstrate that gonadal hormones regulate ERα expression in the SNB target musculature, and suggest that the density of ERα in BC extra-muscle fiber cells determines the estrogen sensitivity of SNB dendrites. Furthermore, to our knowledge, this is the first demonstration that the timing of a sensitive period in sexual differentiation can be regulated by gonadal hormones.

ERα Immunolabeling

It has been known for some time that the BC/LA binds estrogens (Dube et al., 1976), and more recently that the BC muscle expresses ERα (Rudolph and Sengelaub, 2013a). In our previous study describing the developmental expression of ERα in the BC muscle, we found that ERα immunolabeling in BC muscle fibers did not change across ages (Rudolph and Sengelaub, 2013a). The current results demonstrate that ERα expression in BC muscle fibers at P21 is unaltered by castration on P7. This constancy in ERα expression in BC muscle fibers, regardless of age or hormone treatment, suggests that the temporally restricted effects of estrogen on SNB motoneuron morphology are not mediated by muscle fiber ERα.

Our previous work showed that ERα expression in extra-muscle fiber cells is high during the early postnatal period, decreases around P21 and is absent in adulthood (Rudolph and

Sengelaub, 2013a). The current study suggests a potential explanation for the downregulation of ERα-positive extra-muscle fiber cells in the BC, the presence of gonadal hormones. Consistent with the present data, in dorsal root ganglia and skeletal muscles, ER mRNA and ERα mRNA and protein are upregulated after castration, and estradiol treatment in castrates decreases ER levels to those found in gonadally intact animals (Baltgavis et al., 2010; Sohrabji et al., 1994). Future studies should determine if treatment of castrates with estradiol (or androgenic gonadal hormones) prevents the castration-induced upregulation in extra-muscle fiber ERα.

It is possible that other ER isoforms, including ERβ play a role in the estrogenic effects on SNB motoneuron dendrites. In other skeletal muscles, however, ERα appears to be the predominant form of receptor. Skeletal muscle ERα expression is 180 times greater than that of ERβ in humans (Wiik et al., 2003). In mice, ERα expression is more abundant than ERβ (Baltgavis et al., 2010), and in some cases ERβ levels are undetectable (Couse et al., 1997). Furthermore, ERβ in mouse skeletal muscle was not affected by ovariectomy with or without estradiol replacement (Baltgavis et al., 2010).

Target Musculature

BC/LA muscle development requires androgens (Čihák et al., 1970; Tobin and Joubert, 1991). During the first postnatal days the number of myotubes, mononucleate cells, and muscle fibers show an androgen-dependent increase, and androgen exposure during this time is sufficient to maintain BC/LA development through P28 (Hebbeler and Sengelaub, 2003; Tobin and Joubert, 1991). In the present study, BC/LA weights at P28 males castrated at P7 did not differ from those of normal males. Aromatase inhibition in gonadally intact males also does not affect BC/LA muscle growth, demonstrating that androgen-dependent BC/LA development is not mediated by the conversion of testosterone to its estrogenic metabolites (Burke et al., 1999). While androgens promote BC/LA development, estradiol does not appear to have any trophic effects on the BC/LA (Jordan et al., 1995). In fact, estrogen treatment in castrated males inhibits prepubertal increases in BC/LA weight (Hebbeler and Sengelaub, 2003), and BC/LA weight at P28 is decreased in castrated males given estradiol implants at the BC muscle compared to gonadally intact animals (Nowacek and Sengelaub, 2006). In the present study, BC/LA weights

in normal animals treated with estradiol at the muscle from P21-P28 were also smaller than those of untreated normal males. Based on these data, it appears that estradiol not only fails to support normal BC/LA growth, but can produce atrophic effects in gross muscle morphology. Interestingly, while estradiol induces muscle atrophy in gonadally intact males, their SNB dendritic lengths develop normally, and castrated males treated with estradiol at the muscle show SNB dendritic hypertrophy despite having reduced BC/LA weights. This dissociation suggests that the estrogenic support of dendritic growth is not dependent on muscle size per se, but is potentially mediated by other cellular functions of muscle cells or other cell types in the neuromuscular periphery (Nowacek and Sengelaub, 2006).

Motoneuron Morphology

Somata

In normal males, most SNB somal growth occurs during the first four postnatal weeks, and by P28 SNB somata are approximately 75% of their adult sizes (Goldstein et al., 1990). Similar to the BC/LA, SNB somal growth is androgen-dependent (Breedlove and Arnold, 1983a,b; Goldstein and Sengelaub, 1990; Goldstein and Sengelaub, 1994): blocking estrogen synthesis with aromatase inhibition does not alter normal SNB somal growth (Burke et al., 1999) and treating castrates with estradiol through injections or implants fails to masculinize SNB somata (Breedlove et al., 1982; Goldstein and Sengelaub, 1994; Hebbeler et al., 2002; Nowacek and Sengelaub, 2006; Rudolph and Sengelaub, 2013). Consistent with previous findings, in the present study, treatment of castrates with estradiol failed to support SNB somal growth.

Dendrites

In untreated castrates, SNB dendritic lengths were significantly reduced compared with those of normal animals, replicating previous findings (Goldstein et al., 1990; Goldstein and Sengelaub, 1994; Nowacek and Sengelaub, 2006; Rudolph and Sengelaub, 2013a). Our results also confirm the SNB target musculature as the site of action for estrogen-dependent dendrite growth during the early postnatal period (Nowacek and Sengelaub, 2006; Rudolph and Sengelaub, 2013a). Previous work has demonstrated that the BC/LA binds estrogens (Dube et al., 1976) and extra-muscle fiber cells in the BC have been shown to express ERα in a

developmentally transient fashion (Rudolph and Sengelaub, 2013a). The present study further demonstrates that ERα expression in BC extra-muscle fiber cells is also hormone-sensitive. Further, extra-muscle fiber ERα expression in the SNB target muscle coincides with the critical period for estrogen-dependent SNB dendrite growth, suggesting that this type of ERα expression could be important in mediating estrogen sensitivity of SNB motoneuron dendrites (Rudolph and Sengelaub, 2013a). In this study, we show that castration results in a robust upregulation of extra-muscle fiber ERα at P21, and treating castrates with estradiol when ERα is upregulated in extra-muscle fiber cells results in SNB dendritic hypertrophy, despite the limited period of estradiol treatment. In fact, given that estradiol can downregulate its own receptor quite rapidly (Baltgavis et al., 2010), the estrogen-treated castrated animals may have had elevated ERα for an even shorter period during the estradiol treatment. Dendrite lengths of castrates treated with estradiol from P21 to P28 were significantly greater than those of normal males, and this enhanced dendritic growth occurred throughout the arbor. The general hyper-responsiveness of SNB dendrites in castrates to estradiol treatment starting at P21 likely reflects the increase in extra-muscle fiber ERα expression in the SNB target muscle, as previous work has demonstrated that hormone receptor density in skeletal muscle fibers confers hormone sensitivity to motoneuron dendrites (Huguenard et al., 2011). Changes in ERα density in the target musculature would necessarily affect SNB motoneurons as a whole, resulting in the increases in dendritic lengths throughout the arbor we observed. Furthermore, given that alterations in afferent input can result in highly localized changes in SNB dendritic morphology (Hebbeler and Sengelaub, 2003), it is unlikely that the estrogen effects we observed were mediated by changes in specific estrogen-sensitive afferents.

Comparability of HRP Labeling

Previous studies have demonstrated that neither axonal transport of BHRP (Leslie et al., 1991) nor dendritic transport as demonstrated by the rostrocaudal or mediolateral extent of dendritic labeling (Kurz et al., 1990; Goldstein and Sengelaub, 1994) is affected by hormone levels. In the present study, the possibility that hormone manipulations could affect retrograde transport is an important consideration, as such artifact could potentially result in apparent

alterations in dendritic morphology. No difference in rostrocaudal extents of SNB dendrites were observed, but radial extents in untreated castrates were smaller than those of normal males. This result most likely reflects the attenuated growth of SNB dendrites rather than a transport artifact, which (because rostrocaudal extent was not affected) would necessarily have had to occur selectively in the transverse plane. In contrast, no differences in either rostrocaudal or radial extents of dendrites were observed between normal males and estradiol-treated castrates, indicating that the ability of SNB dendrites to transport BHRP out to the most distal, highest order branches was not affected in these groups. Thus, we believe the dendritic labeling across groups was comparable, allowing direct comparisons of dendritic length and distribution across groups.

Mechanisms of Target-Mediated Dendritic Growth

Hormone receptors in the SNB target muscle are important for the development of many features of the SNB system. SNB motoneurons are spared from ontogenetic cell death perinatally by androgen action at androgen receptors (ARs) in the SNB target muscle (Freeman et al., 1996). ERs in the SNB target muscle mediate the estrogen-dependent SNB dendrite growth (Nowacek and Sengelaub, 2006), a developmentally restricted process that coincides with ERα expression in extra-muscle fiber cells in the BC muscle (Rudolph and Sengelaub, 2013a). Furthermore, there is evidence of a causal relationship between hormone receptor expression in the target musculature and the degree of hormone sensitivity of the innervating motoneurons. In rats, dendritic arbors of quadriceps motoneurons are androgen-insensitive, and castration with or without testosterone treatment does not affect dendrite lengths of these motoneurons (Huguenard et al., 2011). When ARs are overexpressed in the skeletal muscle fibers of transgenic rats, dendrites of quadriceps motoneurons become androgen-sensitive: castration results in dendritic atrophy, and dendritic lengths of castrates return to normal lengths with testosterone treatment (Huguenard et al., 2011). The experiments in this Chapter provides a developmental example of the relationship between changes in hormone receptor density in the target muscles of motoneurons and corresponding changes in hormone sensitivity of the dendrites of innervating motoneurons. We have previously demonstrated that the density of ERα expression in extra-muscle fiber cells in the BC muscle coincides with the period of estrogen sensitivity for SNB

dendrites (Rudolph and Sengelaub, 2013a). In this set of experiments, we show that a castration-induced increase in extra-muscle fiber ERα in the developing SNB target muscle results in SNB dendrites that respond to brief estradiol treatment with robust dendritic hypertrophy. Together, these data suggest that in multiple neuromuscular systems, the hormonal sensitivity of motoneuron dendrites is controlled by hormone receptor density in the target muscle.

For estrogen-dependent dendrite growth in the SNB, the relationship between ERα density in the target musculature and estrogen sensitivity of SNB dendrites appears to involve ERα expression in a non-muscle fiber cell type. ERα expression in muscle fibers does not change across development (Rudolph and Sengelaub, 2013a) or in response to removal of gonadal hormones by castration (present Chapter). However, ERα expression in extra-muscle fiber cells in the SNB target muscle coincides with the period of SNB dendrite sensitivity and is also regulated by gonadal hormones. These data suggest that these ERα-expressing extra-muscle fiber cells have a critical role in the estrogen sensitivity of SNB motoneuron dendritic morphology during development. Additionally, non-muscle fiber cells in the SNB target musculature have been suggested to be a critical mediator of androgen-dependent perinatal development of SNB cell number (Niel et al., 2009).

It is clear that an extra-muscle fiber cell type in the SNB target muscle is critical for the masculinization of SNB morphology during development, but the specific identity of this cell type is unknown. While the apparent lack of a basal lamina rules out several cell types (e.g., muscle fibers), other candidates (e.g., fibroblasts) remain. Further, it is not known how estradiol promotes SNB dendrite growth through ERα in extra-muscle fiber cells in the target musculature. A likely mechanism is through the regulation of trophic factors that influence dendritic length. For example, in adulthood, androgens regulate brain-derived neurotrophic factor (BDNF) in SNB motoneurons (Ottem et al., 2007; Verhovshek et al., 2010a), as well as in their target musculature (Verhovshek et al., 2010a), and androgen action at the target musculature regulates BDNF protein in SNB motoneurons (Verhovshek and Sengelaub, 2013). The regulation of BDNF by androgen is critical for SNB dendritic morphology: supplying BDNF to cut axons after peripheral

axotomy restores soma size (Yang and Arnold, 2000) and dendritic length (Yang et al., 2004), while castration results in elevated muscle BDNF levels and dendritic atrophy which can be prevented by blockade of BDNF signaling (Verhovshek and Sengelaub, 2010). Estradiol has been shown to regulate BDNF levels *in vitro* (Krizsan-Agbas et al., 2003) and *in vivo* (Solum and Handa, 2002), and BDNF-ERα coexpression occurs in developing neurons, providing a clear mechanism for estrogen regulation of BDNF (Solum and Handa, 2002). Together, these data suggest that during the early postnatal period, estrogen-dependent SNB dendrite growth could occur through an estrogen-BDNF interaction in the SNB target muscle, and a target-mediated regulation of BDNF levels could be driving changes in SNB dendritic morphology.

CHAPTER 4: Maintenance of ERα in the SNB target muscle maintains estrogen sensitivity of SNB dendrites outside the early postnatal period

BACKGROUND

We have previously demonstrated that the critical period for estrogen-dependent SNB dendrite growth is defined by the presence of ERα expression in BC extra-muscle fiber cells (Rudolph and Sengelaub, 2013a). Further, the potential for SNB dendrites to grow in response to estrogen treatment appears to be determined by the density of extra-muscle fiber ERα in the SNB target muscle. When ERα is downregulated in the SNB target muscle, the critical period for estrogen-dependent dendrite growth closes, rendering SNB dendritic morphology insensitive to estrogens. However, early postnatal castration results in a marked upregulation of this extra-muscle fiber cell ERα in the BC muscle. If this castration-induced ERα upregulation is maintained outside of early development, the mechanism for estrogen-dependent dendrite growth is present, and dendritic morphology should respond to estradiol treatment allowing for estrogen-dependent SNB dendrite growth to occur outside the critical period for estrogen-dependent SNB dendrogenesis. In this experiment, we test the hypothesis that maintenance of extra-muscle fiber cell ERα in the BC permits estrogen-dependent SNB dendrite growth outside of early development, when SNB dendrites are typically insensitive to estrogens.

MATERIALS AND METHODS

Animals. Untimed pregnant rat dams (Sprague-Dawley; Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle, with unlimited access to food and water. Litters were culled to eight pups when necessary, retaining males preferentially. To preserve ERα expression in the BC muscle (Rudolph and Sengelaub, 2013b), one group of males was bilaterally castrated on P7, and remained untreated until P28, when they had estradiol implants (see below) placed at the left BC muscle, remaining until P49 (n = 5); another group of males castrated on P7 were left untreated (n = 4). Another group of males was castrated on P28, after ERα downregulation, and received daily systemic injections of estradiol benzoate (0.1 mg estradiol in sesame oil, sc; n = 3) from P28 to P49. Age-matched intact control males (n = 5) were also used (overall n = 17). All procedures were carried out in accordance with the NIH *Guide for the Care and Use of*

Laboratory Animals and approved by the Bloomington Institutional Animal Care and Use Committee.

Implants. To restrict hormone treatment to the SNB target musculature, we used our previous method (Nowacek and Sengelaub, 2006; Rudolph and Sengelaub, 2013a; Rudolph and Sengelaub, 2013b) to deliver estradiol locally to the BC muscle. This treatment produces alterations in motoneuron morphology that can be directly ascribed to local rather than systemic effects; furthermore, the treatment has no effect on the typical development of SNB motoneurons, and no effect on dendritic labeling or tracer transport (Nowacek and Sengelaub, 2006; Rudolph and Sengelaub, 2013a; Rudolph and Sengelaub, 2013b). Small (0.85 mm^3) implants impregnated with estradiol (Steraloids, Newport, RI) were constructed by mixing crystalline estradiol with Silastic adhesive (Dow-Corning, Auburn, MI) and compressing the mixture between two glass slides (lightly greased with petroleum jelly) separated by a spacer (0.85 mm thick), and allowed to cure. The cured Silastic was cut into 1 x 1 mm pieces (total volume 0.85 mm^3) and coated on five sides with acrylic. The amount of estradiol in each implant (0.1 mg) was similar to that used previously; this dosage does not produce systemic effects, and supports SNB dendritic growth (Nowacek and Sengelaub, 2006).

Horseradish peroxidase conjugated to the cholera toxin β subunit (BHRP; List Biological, Campbell, CA) was used to retrogradely label SNB motoneurons innervating the BC muscle. Previous studies have demonstrated that BHRP labeling permits sensitive detection and quantitative analysis of SNB somal and dendritic morphologies (Kurz et al., 1986; Goldstein and Sengelaub, 1990; Hebbeler and Sengelaub, 2003). SNB motoneuron morphology was examined at P49, (when SNB dendrites have reached their adult lengths; Goldstein et al., 1990). At P47, animals were anesthetized, the perineal muscles exposed, and BHRP unilaterally injected (0.5 µl; 0.2% solution) into the left BC muscle, the same muscle for those males that received muscle implants. Forty-eight h after BHRP injection, a period that ensures optimal labeling of SNB motoneurons (Kurz et al., 1986; Goldstein et al., 1990), animals were given an overdose of urethane (0.5 ml/100 g body weight) and perfused intracardially with saline followed by cold 1% paraformaldehyde/1.25% glutaraldehyde fixative. Lumbar spinal cords were removed, postfixed

in the same fixative for 5 h, and transferred to sucrose phosphate buffer (10% w/v, pH = 7.4) overnight for cryoprotection. Spinal cord segments were then embedded in gelatin, frozen, and sectioned transversely at 40 μm; all sections were collected into four alternate series. For visualization of BHRP, the tissue was immediately reacted using a modified tetramethyl benzidine protocol (TMB; Mesulam, 1982), mounted on gelatin-coated slides, counterstained with thionin, and cover-slipped with Permount. BC/LA muscles were removed at perfusion and weighed to evaluate potential treatment effects on gross muscle development.

The number of BHRP-filled motoneurons was assessed in all sections through the entire rostrocaudal extent of the SNB for all animals. Counts of labeled motoneurons in the SNB were made under brightfield illumination, where somata and nuclei could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed. Estimates of the total number of labeled SNB motoneurons were obtained using the optical dissector method outlined by Coggeshall (1992) and a procedure similar to that of West and Gunderson (1990). This method yields an unbiased count of SNB motoneurons (Raouf et al., 2000). Counts were made at 500X; motoneuron somata could be easily visualized in multiple focal planes. BHRP-labeled motoneurons were counted as their somata first appeared in focus while focusing through the *z* axis, and labeled somata in the first focal plane (i.e., “tops”) were not counted. For each animal, counts were derived from sections spaced at 160 μm intervals uniformly distributed through the entire rostrocaudal extent of the SNB. Within each section, all labeled somata within the SNB were counted. Estimates of the total number of labeled SNB motoneurons were then obtained by correcting for percentage of the tissue sampled.

The cross-sectional soma area of BHRP-labeled motoneurons was measured using a video-based morphometry system (Stereo Investigator; MBF Bioscience, Inc.) at a final magnification of 1350X. Soma areas within each animal were averaged for statistical analysis. The optical density of labeled somata was also measured under brightfield illumination to confirm equivalence of BHRP labeling density.

For each animal, dendritic lengths in a single representative set of alternate sections were measured under darkfield illumination. Beginning with the first section in which BHRP-

labeled fibers were present, labeling through the entire rostrocaudal extent of the SNB dendritic field was assessed in every other section (320 μm apart) in three dimensions using a computer-based morphometry system (Neurolucida; MBF Bioscience, Inc.; final magnification 250X) to yield both composite illustrations of the arbor and measurements of individual fiber lengths. All BHRP-labeled fibers were drawn regardless of location, size, or contiguity with labeled cell bodies to ensure a complete assessment of dendritic length. Because the entire rostrocaudal range of the SNB dendritic field in each animal was sampled, this method allows for a complete assessment of SNB dendrites in both the transverse and horizontal planes. Average dendritic arbor per labeled motoneuron was estimated by summing the measured dendritic lengths of the series of sections, multiplying by two to correct for sampling, then dividing by the total number of labeled motoneurons in that series. This method does not attempt to assess the actual total dendritic length of labeled motoneurons (Kurz et al., 1991), but has been shown to be a sensitive and reliable indicator of changes in dendritic morphology in normal development (Goldstein et al., 1990), after hormonal or surgical manipulation (Kurz et al., 1986; Goldstein et al., 1990; Kurz et al., 1991; Goldstein and Sengelaub, 1994; Goldstein et al., 1996; Hays et al., 1996; Hebbeler and Sengelaub, 2003), due to dendritic interactions (Goldstein et al., 1993), or after NMDA receptor blockade (Hebbeler et al., 2002).

Injections of BHRP into the left BC successfully labeled ipsilateral SNB motoneurons in all animals in a manner consistent with previous studies (Kurz et al., 1986; Goldstein and Sengelaub, 1990; Goldstein and Sengelaub, 1994; Burke et al., 1997; Burke et al., 1999; Fig. 4.1). SNB motoneurons displayed their characteristic multipolar morphologies, with dendritic arbors projecting ventrolaterally, dorsomedially, and across the midline into the area of the contralateral SNB (Fig. 4.1).

RESULTS

Soma size. The size of SNB somata at P49 differed significantly across groups [$F(3,13) = 3.96$, $p < 0.05$; Fig. 4.2]. The mean cross-sectional area of SNB somata in intact control males (741.21 ± 22.93 μm^2) was typical. The somata of untreated castrates (697.86 ± 54.19 μm^2) were not

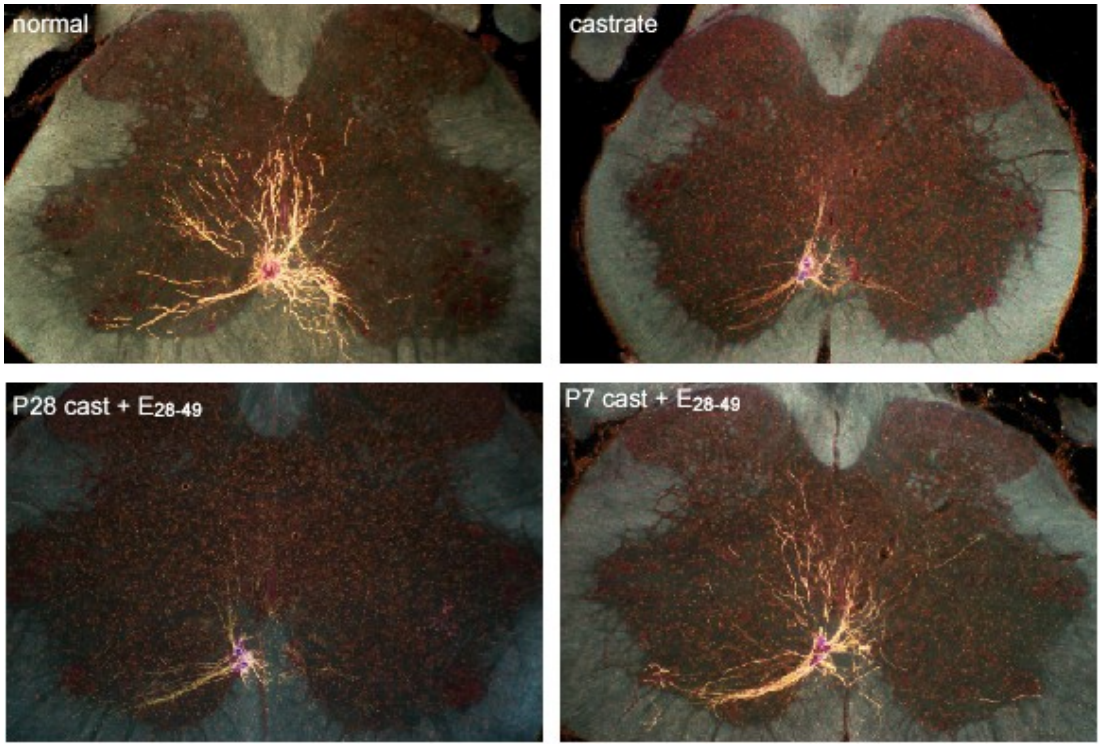

Figure 4.1. Darkfield digital images of transverse sections through the lumbar spinal cord of male rats at P49 showing labeled SNB motoneurons following injection of BHRP into the left BC muscles of a normal male (normal; top left), a male castrated on P7 and left untreated (castrate; top right), a male castrated at P28 and treated with estradiol from P28 to P49 (P7 cast + E28-49; bottom left) , and a male castrated on P7 and treated with estradiol from P28 to P49 (P7 castrate + E28-49; bottom right).

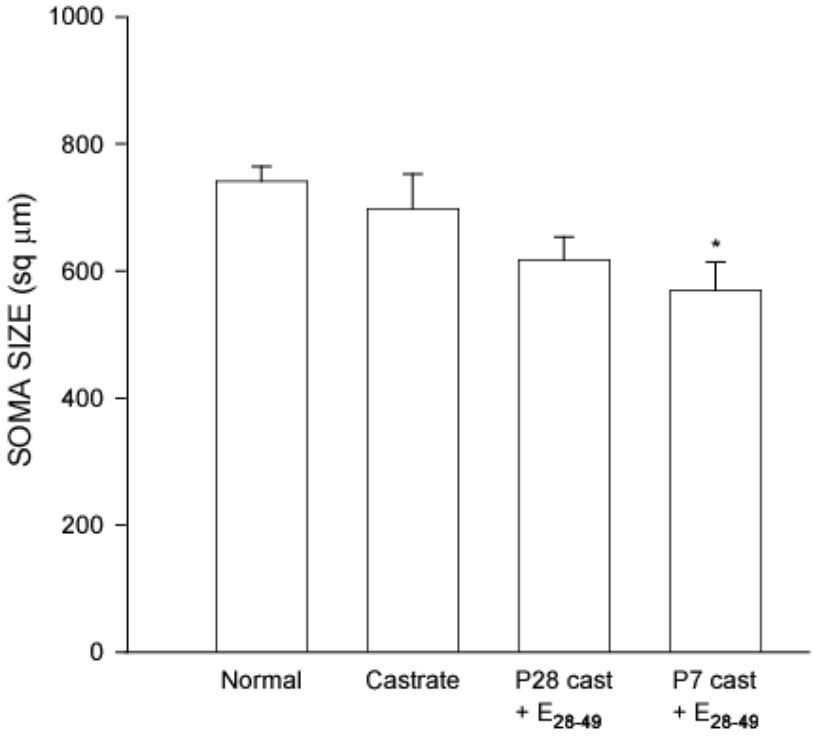

Figure 4.2. Soma areas of SNB motoneurons at P49 for normal males, animals castrated on P7 and left untreated (castrate), animals castrated at P28 and treated with estradiol from P28 to P49 (P7 cast + E28-49), and animals castrated on P7 and treated with estradiol from P28 to P49 (P7 castrate + E28-49). Bar heights represent means ± SEM for three to five animals per group. * significantly different from normal males.

different than those of normal animals. Animals castrated on P7 and treated with estradiol from P28 to P49 (568.88 ± 44.84 μm^2) had somata that were significantly reduced (23%) compared to those of normal animals (LSD, $p < 0.05$), and somata of P28 castrates treated with estradiol implants from P28-P49 (638.18 ± 13.77 μm^2) had somata that were marginally significantly smaller (reduced 17%) than those of intact control males (LSD, $p = 0.07$).

Dendritic lengths. The overall length of SNB dendrites significantly differed across groups [$F(3,13) = 5.50$, $p < 0.05$; Fig. 4.3]. SNB dendritic length in intact control males was typical (4266.52 ± 519.32 μm). Dendritic lengths in castrates were approximately 47% shorter (2278.51 ± 658.72 μm) than those of intact control males (LSD, $p = 0.06$). Males castrated on P28 and treated with estradiol from P28-P49 had SNB dendritic arbors (1990.78 ± 278.85 μm) that were reduced 53% compared to those of intact control males (LSD, $p = 0.05$), and were not different than those of untreated castrates (LSD, $p < 0.05$). P7 castrates treated with estradiol from P28-P49 had dendritic lengths that were significantly greater than those of untreated castrates (LSD, $p < 0.05$, increased 159%) and P28 castrates treated with estradiol from P28-P49 (LSD, $p < 0.05$, increased 164%).

DISCUSSION

In this experiment we demonstrated that the early postnatal castration-induced maintenance of ERα in the SNB target muscle allows for estrogen-dependent SNB dendrite growth outside the early postnatal period. This is the first demonstration that SNB dendritic morphology is estrogen-sensitive after the first four postnatal weeks. These results further demonstrate that the timing of early gonadal hormone exposure determines the sensitivity of the system to gonadal hormones later in development.

Somata

At P49, SNB somata of P28 castrates treated with estradiol from P28 to P49 were not different than those of intact animals. The majority of somal growth occurs during the first four postnatal weeks (Goldstein et al., 1990), potentially explaining why castration at P28 did not affect the size of SNB somata at P49. However, animals castrated on P7 and treated with

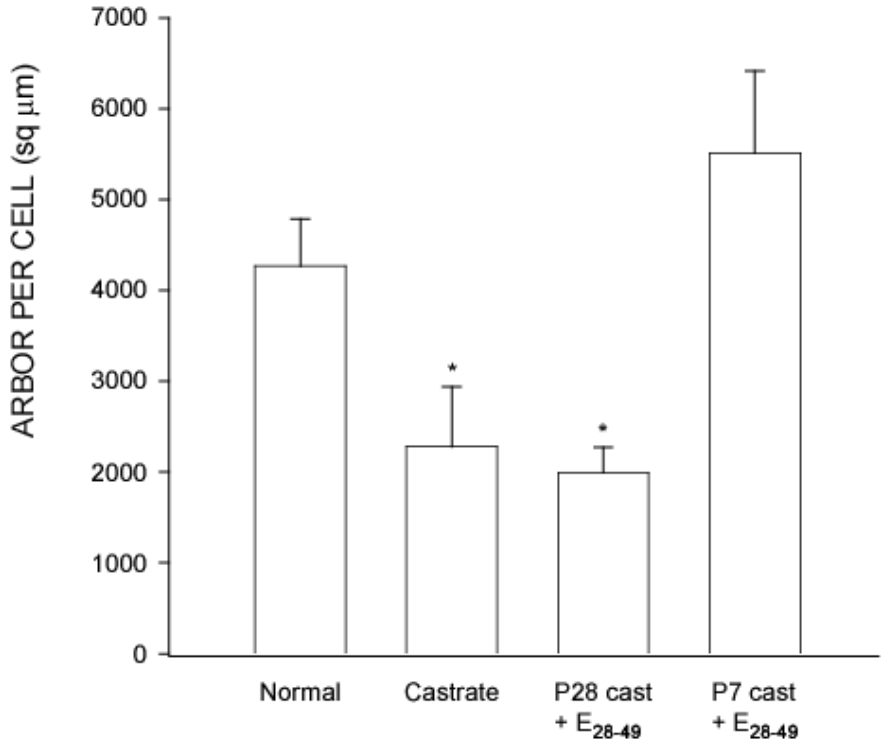

Figure 4.3. Dendritic lengths expressed as length of arbor per labeled motoneuron of SNB motoneurons at P49 for normal males, animals castrated on P7 and left untreated (castrate), animals castrated at P28 and treated with estradiol from P28 to P49 (P7 cast + E28-49), and animals castrated on P7 and treated with estradiol from P28 to P49 (P7 castrate + E28-49). Bar heights represent means ± SEM for three to five animals per group. * significantly different from P7 castrates treated with estradiol from P28 to P49.

estradiol from P28 to P49 had somata that were significantly smaller than those of intact males. This effect can be explained due to the absence of androgen exposure from P7 to P49, as soma size is strictly androgenically regulated (e.g., Goldstein and Sengelaub, 1994), and also the effects of local estradiol treatment at the SNB target muscle, which has been shown to decrease soma sizes in castrated animals (Nowacek and Sengelaub, 2006; Rudolph and Sengelaub, 2013a; Rudolph and Sengelaub, 2013b). There was a trend towards a castration-induced decrease in SNB somata in all castrate groups, but untreated castrates had somata that were no different than those of gonadally intact males. This can be explained by the greater variability in somata size in untreated castrates, and also the small number of animals (3-5) used per group in the analysis. If more untreated castrates were added to the group, we expect that the typical castration-induced decrease in SNB soma size would be observed. Importantly, the critical comparison for this experiment is SNB dendrite length, not soma size, and the patterns of change in SNB dendrites in response to castration and estradiol treatment follow the expected results. Further, it has been established that there is a disconnect between SNB soma size and dendritic length (e.g., Rudolph and Sengelaub, 2013a), so the failure to obtain a castration-induced decrease in SNB somata size does not confound the conclusions of this experiment.

Dendrites

Results demonstrated that preservation of muscle ERα via early castration resulted in estrogen-sensitive SNB dendrites outside of the critical period for estrogen-dependent SNB dendrite growth. When males were castrated on P7, estrogen treatment from P28-P49 resulted in robust dendritic growth, and dendrite lengths of P7 castrates treated with estradiol from P28-P49 were no different than those of age-matched intact control males. However, when males were castrated on P28, after ERα in the muscle had downregulated, estradiol treatment from P28-P49 had no effect on SNB motoneuron dendrite growth, and dendrites of these animals were no different than those of untreated castrates. Together, these results demonstrate that early castration creates estrogen-sensitive SNB dendrites by maintaining ERα in the muscle, allowing for estrogen-dependent dendrogenesis outside the critical period for SNB dendrite growth in response to estrogens. Not only do SNB dendrites respond to estradiol when ERα is maintained

in the SNB target muscle, but they do so by growing during a time when SNB dendrites typically are also insensitive to estrogens. Previous work has demonstrated that a castration-induced upregulation of ERα in the SNB target muscle results in hypersensitivity of SNB dendrites to estradiol during the early postnatal period, but this is the first demonstration that SNB dendrites grow in response to estrogens after P28. The current findings build upon earlier data establishing the relationship between extra-muscle fiber ERα in the BC and SNB dendrite estrogen sensitivity (Rudolph and Sengelaub, 2013b), and demonstrate that the castration-induced upregulation of ERα in the muscle extends the critical period for estrogen-dependent SNB dendrite growth.

CONCLUSIONS

These findings further substantiate the SNB target muscle as the critical site of action for the influence of gonadal hormones on SNB motoneuron morphology. Hormone receptor density has been shown to confer hormone sensitivity to motoneurons previously (Huguenard et al., 2011). These findings extend the importance of the muscle-based receptor—motoneuron sensitivity relationship to demonstrate the mechanism for shifting a critical period for estrogen-dependent SNB dendrite growth: is hormone receptor expression in the SNB target muscle. The current study demonstrated that estrogen-dependent dendrite growth occurs outside of P28, but it is unclear how long SNB dendrites remain estrogen sensitive after castration on P7. We have shown that this early castration upregulates extra-muscle fiber ERα at P21, but it is unknown if this castration-induced upregulation is stable for an extended period of time. Future studies should determine the time course of castration-induced estrogen sensitivity of SNB motoneuron dendrites by delaying post-castration estradiol treatment until adulthood to see if the critical period for estrogen-dependent SNB dendrite growth can be extended indefinitely.

Castration prior to the developmental downregulation of extra-muscle fiber ERα maintains the sensitivity of SNB dendrites, but it is unclear why the developmental downregulation of ERα occurs during typical development. We have posited that high estrogen levels during early postnatal life drive down ERα expression in the SNB target muscle, but this hypothesis has not been directly tested. Castration prevents the developmental decline in ERα, demonstrating that

the removal of the gonad triggers a change in ERα in the muscle, which does not eliminate the possibility that other gonadal steroids are responsible for alterations in ERα expression in the SNB target muscle. Testosterone or its androgenic metabolite, dihydrotestosterone could also be involved in the regulation of ERα in the muscle, and castration-induced changes in muscle ERα could be mediated by androgens, not estrogens. Additionally, sex hormone binding globulin (SHBG) production occurs in the testes, and removal of the gonads by castration could alter SHBG levels thereby changing circulating levels of androgens and estrogens to modulate muscle ERα levels. SHBG is present in developing rat skeletal muscle (Becchis et al., 1996), and has been shown to mediate activation of AR and ERs (Kahn et al., 2008), indicating that changes in muscle ERα could be occurring directly or indirectly through the regulation of SHBG.

Regardless of the mechanism of castration-induced changes in extra-muscle fiber ERα, it is clear that ERα in the BC mediates estrogen-dependent dendrite growth, and can do so outside of early development. This finding is important for understanding the relationship between developmental gonadal hormone profiles and neural sensitivity to gonadal hormones in later development and adulthood. Although castration does not capture an ecologically relevant alteration in circulating gonadal hormone levels, it is likely that more subtle, natural changes in gonadal hormones during masculinization will affect the sensitivity of gonadal hormone-sensitive structures later in development. Future studies should address how natural shifts in androgens and estrogens can prime the neuraxis for hyper- or hyposensitivity of neural structures to these same hormones during adolescence and adulthood.

CHAPTER 5: ERα is not localized within satellite cells in the SNB target muscle during early development

BACKGROUND

As demonstrated in previous Chapters, the transient period of peripherally-mediated, estrogen-dependent SNB dendrite growth is defined by a change in ERα expression at the target musculature. ERα expression in extra-muscle fiber cells is greatest during the period of estrogen-dependent dendrite growth, but declines significantly, and is low or absent when SNB dendrites are insensitive to estrogens (Rudolph and Sengelaub, 2013a; Chapter 2). Furthermore, restricting estradiol treatment at the BC muscle to the period when extra-muscle fiber cell ERα expression is greatest results in complete masculinization of SNB dendrites (Rudolph and Sengelaub, 2013a; Chapter 2). The location of expression within the BC muscle is particularly important: ERα expression in extra-muscle fiber cells parallels the estrogenic sensitivity of SNB dendrites, whereas there is no change in muscle fiber ERα across development. Additionally, just as gonadal hormones regulate SNB dendritic morphology, they alter BC extra-muscle fiber cell ERα expression, which is dramatically upregulated following castration (Rudolph and Sengelaub, 2013b; Chapter 3). However, no castration-induced changes in muscle fiber ERα are observed, suggesting that ERα expression in extra-muscle fiber cells but not within muscle fibers mediates the estrogen sensitivity of SNB dendrites (Rudolph and Sengelaub, 2013b; Chapter 3). Also, the castration-induced upregulation of BC extra-muscle fiber ERα preserves estrogen sensitivity of SNB dendrites, and one week of estradiol treatment is sufficient to fully masculinize SNB dendritic morphology when the period of estrogen treatment coincides with heightened BC extra-muscle fiber ERα expression (Rudolph and Sengelaub, 2013b; Chapter 3). Together, these data demonstrate that the critical period for estrogen-dependent SNB dendrogenesis is defined by ERα expression in BC extra-muscle fiber cells, and these ERα-expressing BC cells appear to mediate estrogen sensitivity of SNB dendrites during the early postnatal period.

This is not the first example of hormone receptor expression in extra-muscle fiber cells controlling masculinization of SNB features. The sex difference in SNB cell number occurs through the actions of perinatal androgens: in males, motoneurons are spared from cell death by androgen action through androgen receptors (ARs), presumably in the BC muscle (Fishman et al., 1996). In females without sufficient androgens, more motoneuron death occurs, resulting in a

smaller, feminine SNB. ARs are required for the rescue of SNB motoneurons from death, and the location of AR in the BC muscle appears to be critical for masculinization of SNB cell number as well. However, in a transgenic animal expressing androgen receptors (ARs) solely within muscle fibers, the SNB motoneurons were female in number and size, demonstrating that AR expression within muscle fibers is not sufficient to mediate masculinization of the SNB (Niel et al., 2009). Recent evidence shows that AR-expressing satellite cells are present in the LA during the period when androgens act at the SNB target muscle to spare motoneurons from cell death (Swift-Gallant and Monks, 2013). Additionally, satellite cells in the SNB target muscle are regulated by androgens: testosterone treatment increases satellite cell number in the LA, implicating these cells as an androgen target during early development (Niel et al., 2008). Similar to the role of AR in androgen regulation of SNB motoneuron number, ERα expression that appears to mediate estrogen-dependent masculinization of SNB dendrites occurs in BC non-muscle fibers. Because ERα expression has previously been demonstrated in satellite cells of skeletal muscle (Kalbe et al., 2007), we hypothesized that ERα expression in satellite cells during the period of estrogen-dependent masculinization of SNB dendrites will occur.

MATERIALS AND METHODS

Animals. Untimed pregnant rat dams (Sprague-Dawley, Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle, with unlimited access to food and water. When pregnant females gave birth (day of birth = P1), pups were sexed and litters were culled to eight pups when necessary, retaining males preferentially. All procedures were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Bloomington Institutional Animal Care and Use Committee.

Immunohistochemistry. Immunohistochemistry was performed largely according to the methods used previously (Rudolph and Sengelaub, 2013a; Rudolph & Sengelaub, 2013b; Chapters 2-3). At P14, when extra-muscle fiber ERα levels are high (Rudolph and Sengelaub, 2013a), animals (n = 5) were weighed, given an overdose of urethane (0.25 g/100 g of body weight), and perfused transcardially with 0.9% saline followed by cold 4% paraformaldehyde in

0.1M phosphate buffer (pH = 7.4). The BC/LA muscles were removed and postfixed in the same fixative for 24 h and transferred to 30% sucrose in 0.1M phosphate buffer for a minimum of 24 h of cryoprotection. The BC/LA was cut horizontally into 12 µm sections on a cryostat at -16°C. Sections were thaw-mounted onto gelatin-coated slides, and stored at -16°C. For immunohistochemical processing, slides were brought to room temperature, and rinsed 3 x 5 min in phosphate buffered saline (PBS, pH 7.4). After rinsing, a hydrophobic border (Super Pap Pen, Ted Pella, Inc., Redding, CA) was created around each tissue section. All incubations occurred in a humidified chamber and were performed by pipetting 200 µl of solution onto each section.

Sections were incubated for 45 min at room temperature in blocking solution containing 10% normal goat serum (NGS; Vector Laboratories, Inc., Burlingame, CA) and 10% normal horse serum (NHS, Vector Laboratories, Inc.) 0.24% Triton X-100 in PBS. Sections were then incubated for 48 h at 4ºC in 4% NGS and 4% NHS with 0.276% Triton X-100 in PBS containing an antibody directed against the last 14 amino acids of the rat ERα (C1355, 1:1000; Millipore, Temecula, CA) and an antibody that labels satellite cells (PAX7, concentrate antiserum, 1:200, Developmental Studies Hybridoma Bank, Iowa City, IA). Slides were rinsed with PBS, and avidin-biotin (Vector), and washed with PBS, followed by a two hour incubation in secondary antibodies (goat anti-rabbit Alexa Fluor 488 $F(ab')_2$ fragments (H+L), 1:200; Invitrogen, Eugene, OR; rat adsorbed affinity purified biotinylated horse anti-mouse IgG, 1:200). Slides were washed 3 x 5 minutes in PBS and incubated for 1 hour in Cy3-conjugated streptavidin (1:5000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Slides were washed 3 x 5 min in PBS, briefly dried, and coverslipped with Vectashield hard set mounting medium with DAPI (H-1500, Vector Laboratories). Slides were laid flat to dry for 5 minutes, sealed with nail polish (Deep Purple, Target, Minneapolis, MN), and dried at room temperature for 10-15 minutes, and laid flat to dry overnight at 4°C, and then stored in black boxes at 4C until microscopic analysis. To control for nonspecific staining, control sections incubated without both primary antibodies were generated and demonstrated no immunostaining.

Microscopic Analysis. Images were captured using a Leica SP5 laser scanning inverted 5-channel confocal microscope (Leica Microsystems, Buffalo Grove, IL). For each animal, 2-3

sections (mean = 2.2) and 3-6 fields (mean = 4.5) were sampled. A minimum of 15 satellite cells were required for a field to be included in the analysis. Initial analysis of images was performed using LAS-AF LITE software, and images were exported to Photoshop (Adobe) for further analysis. ERα label (green; FITC) and PAX7 label (red; Cy3) were clearly visible in each field (Fig. 5.1). All PAX7-labeled cells were counted for each captured image, and partial cells were excluded from the total number of PAX7-positive cells for each field. ERα label and PAX7 label clearly occupied distinct space in the captured images. To ensure that ERα and PAX7 labels were not colocalized, stacks of images from the z-axis were compiled to create a 3-dimensional image, and these z-stacks demonstrated no colocalization of ERα and PAX7.

RESULTS

In an average of 145.6 ± 6.14 (SEM) PAX7-labeled satellite cells per animal, no colocalization of ERα and PAX7 was observed (Fig. 5.2).

DISCUSSION

These data demonstrate that at P14, ERα expression does not occur within satellite cells in the BC muscle, as no evidence of PAX7-ERα colocalization was observed. This suggests that the ERα-expressing extra-muscle fiber cells in the BC that are critical for estrogen-dependent SNB dendrite growth are not satellite cells. It is possible that colocalization of ERα and PAX7 occurs in the SNB target muscle, and it was not observed due to undersampling. Previous work using double-labeled immunofluoresence in the SNB target muscle concluded an absence of colocalization of AR and fibroblast label in two experimental groups sampling an average of 94.5 and 69.5 cells of interest (Swift-Gallant and Monks, 2013; Fig. 5.1). In this experiment significantly more cells of interest were analyzed and no colocalization occurred. For this reason, I am confident in concluding that these data demonstrate that the label of interest (ERα) is not localized within the cells of interest (satellite cells).

Because the ERα-extra-muscle fiber cells in the BC muscle are not satellite cells, future studies should determine what cell type they are. Fibroblasts are one candidate cell type, as they

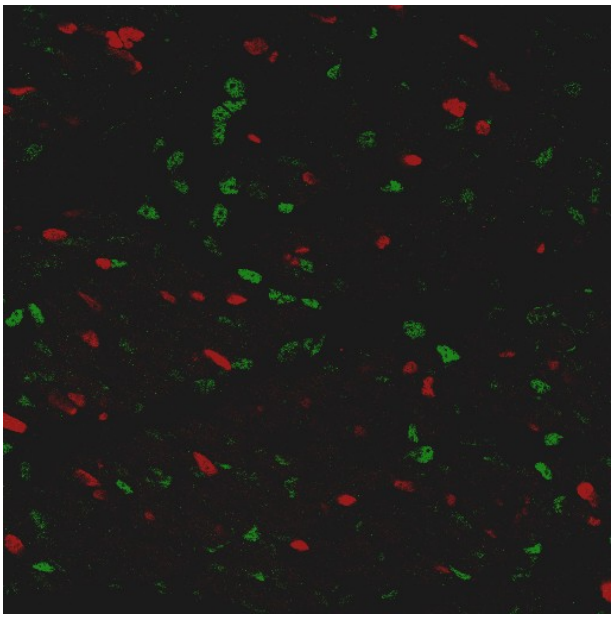

Figure 5.1. Representative confocal image of BC muscle at P14 displaying ERα (green) and PAX7 immunolabeling (red). ERα expression does not occur within satellite cells (identified by PAX7 labeling).

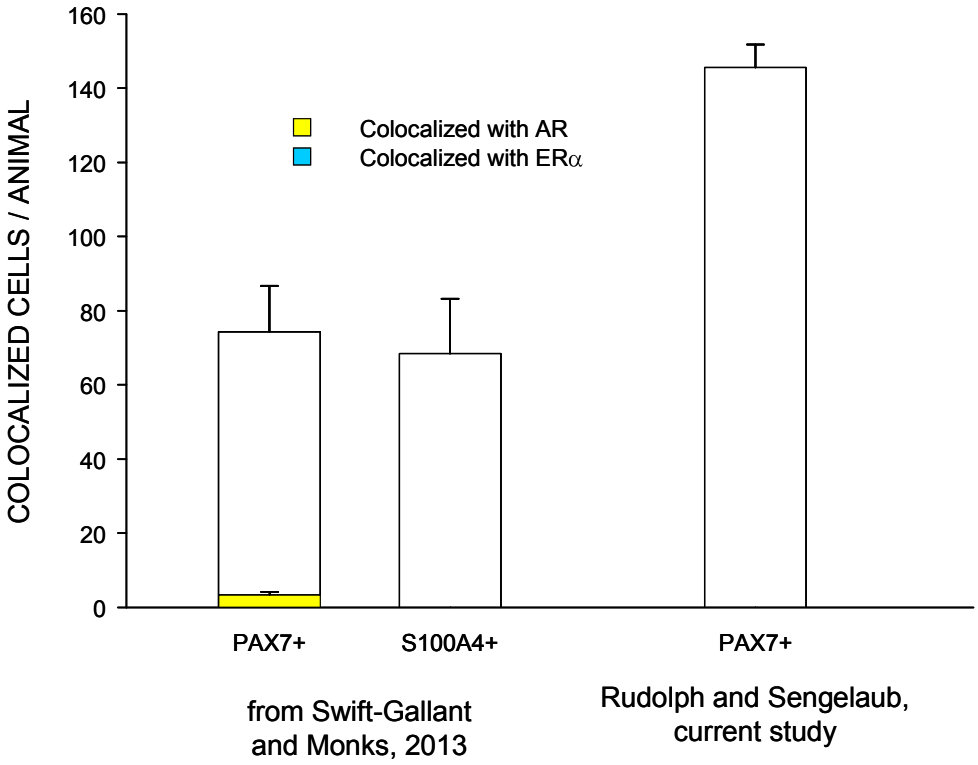

Figure 5.2. Combined data from the current study (right) and a previous study (left bars) assessing colocalization of ERα and AR (respectively) in the developing SNB target muscle. AR is localized within satellite cells (PAX7+; yellow) in the SNB target muscle, but not in fibroblasts (S100A4+ cells). ERα was not localized within satellite cells in the developing SNB muscle.

are found in higher levels during development, when we have observed extra-muscle fiber ERα. While it is promising that the extra-muscle fiber ERα expression profile might parallel that of fibroblast expression in the developing muscle (e.g., high during early development, decreasing through late development), it is unknown whether the developmental downregulation of extra-muscle fiber ERα is due to the loss of ERα-expressing cells in the muscle, or a decrease in ERα without a change in cell type expressing ERα. Other data suggesting fibroblasts are a good extra-muscle fiber cell type candidate demonstrate that AR expression occurs within fibroblasts of the SNB target muscle in mice (Johanson et al., 2007) and rats (Monks et al., 2004). How castration affects fibroblast profiles or if SNB target muscle fibroblasts express ERα should be assessed to determine if fibroblasts remain a likely candidate cell type for the ERα-expressing cells that mediate estrogen-dependent SNB dendrite growth.

Another possible cell type that could express ERα during early SNB development is terminal Schwann cells. Terminal Schwann cells are present in muscle in high densities at the neuromuscular junctions and play an important role in neuromuscular physiology. Similar to ERα-expressing extra-muscle fiber cells, terminal Schwann cell densities are very high in the developing SNB target muscle and decrease with age (Love and Thompson, 1998). Further, terminal Schwann cells are sensitive to gonadal hormone manipulation: castration reduces terminal Schwann cell density, and testosterone treatment restores terminal Schwann cell levels to those of gonadally intact animals both in adulthood (Lubischer and Bebinger, 1999) and development (Jordan and Williams, 2001). However, AR is not found in terminal Schwann cells of the SNB target muscle (Monks et al., 2004), so androgenic regulation of terminal Schwann cell number must occur indirectly. Further, ERα expression in terminal Schwann cells has not been demonstrated. Future studies should investigate the potential for terminal Schwann cells in BC muscle to express ERα.

Adipocytes are another cell type that could be involved in estrogen-dependent SNB dendrite growth. Adipocytes have been shown to express ERα in human fetal adipose tissue (Velickovic et al., 2014) suggesting the possibility that ERα can be expressed in adipocytes in rat skeletal muscle, although this remains to be determined. Gonadal hormones regulate many

features of adipocyte structure and function. For example, in adult mice, castration results in changes in adipocyte AR expression (Floryk et al., 2011). While the effects of castration on ERα expression in adipocytes are unknown, the potential for adipocytes to be the ERα-expressing extra-muscle fiber cells involved in estrogen-dependent dendrite growth remains.

Although the current data have ruled out satellite cells in the SNB target muscle as the extra-muscle fiber cells critical for estrogenic dendrogenesis during early postnatal development, fibroblasts and terminal Schwann cells, and to a lesser extent, adipocytes, are promising potential extra-muscle fiber cell types. Future studies should assess the potential localization of ERα within these muscle cell types and should determine if these cell types are sensitive to gonadal hormones and indeed are the site of action for estrogen-dependent SNB dendrite growth.

CHAPTER 6: Effects of early castration and estradiol treatment on BDNF levels in SNB motoneurons and the SNB target musculature

BACKGROUND

During early development, SNB motoneuron dendrites undergo robust dendritic growth in response to estrogens. Estrogen-dependent SNB dendrite growth is transient (Nowacek and Sengelaub, 2006), and restricted to the early postnatal period when ERα is expressed in high densities in extra-muscle fiber cells of the SNB target muscle (Rudolph and Sengelaub, 2013a, 2013b; Chapter 2). This ERα expression profile is sensitive to gonadal hormones, and castration results in a marked elevation of ERα with a coincident increase in the estrogen responsivity of SNB dendrites (Rudolph and Sengelaub, 2013b; Chapter 3). Although we understand the relationship between ERα in the SNB target muscle and the estrogen sensitivity of SNB motoneuron dendrites, the mechanisms responsible for these development- and castration-induced changes in ERα remain unknown. Conversely, the molecular mechanisms of androgen-dependent influences on SNB dendrites in adulthood have been investigated and involve brain-derived neurotrophic factor (BDNF), so it is possible that the same mechanisms that underlie the maintenance of androgen-dependent of adult SNB motoneuron morphology also control estrogen-dependent changes in SNB motoneurons during development. Further, BDNF has been shown to play a role in hormone-mediated masculinization of the SNB during early development (motoneuron number; Xu et al., 2001), and estrogen-BDNF interactions regulate dendritic morphology across the neuraxis. These data suggest that estrogen can regulate BDNF in the same manner as androgens in the SNB system, and demonstrate that estrogen-dependent dendrite growth could be mediated by changes in muscle BDNF. To determine if BDNF is affected by alterations in gonadal hormones during the period of estrogen-dependent dendrite growth, we assessed BDNF levels in SNB neuromuscular system after castration and estradiol treatment during the early postnatal period, when estradiol treatment results in robust SNB dendrogenesis. BDNF levels were measured in SNB motoneurons (using immunohistochemistry) and the SNB target muscle (using ELISA) in castrates, castrates treated with estradiol implants placed interscapularly, castrates treated with estradiol implants at the SNB target muscle, castrates treated with blank implants at the SNB target muscle, and intact control males.

Experiment 1

MATERIALS AND METHODS

BDNF Immunohistochemistry. On postnatal day 7 (P7) male rats were bilaterally castrated under isoflurane anesthesia. Some castrates received estradiol implants (0.1 mg) at the left BC muscle (*n* = 6), some received similar estradiol implants placed in the interscapular region (*n* = 4), some received implants without hormone that were placed at the left BC muscle (blank implants; *n* = 5) and some were left untreated (*n* = 5). Another group of males remained gonadally intact (*n* = 4). On P21, animals were weighed, overdosed with urethane (0.25 g/100 g body weight), and perfused intracardially with 0.1 M sodium phosphate buffer followed by cold 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH = 7.2). The lumbar segment (L5-S1) of cord was postfixed individually in the same fixative for 18 h and then transferred to 30% sucrose in 0.1M sodium phosphate buffer (pH = 7.4) overnight for cryoprotection. The following day, spinal cords were sectioned horizontally at 30 µm and stored in 0.1 M sodium phosphate buffer. Sections were rinsed with 0.01M PBS and incubated 30 min in 0.5% hydrogen peroxide in a blocking solution containing 1% normal goat serum and 0.1% Triton X-100 in 0.01M PBS, followed by 1 h in blocking solution without hydrogen peroxide. Sections were then incubated 48 h at 4°C in rabbit anti-BDNF primary antibody (1:1000, AB1779; Millipore, Temecula, CA) followed by 24 h incubation in biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA). Antibody signal amplification was achieved with an ABC reaction (Vector Standard Elite Kit) and visualized with 0.035% diaminobenzidine and 0.006% H_2O_2. Sections were mounted onto gelatin-coated slides, defatted, and coverslipped.

A semi-quantitative method of categorizing cells as intensely, moderately, or lightly immunolabeled was used (Osborne et al., 2007; Verhovshek et al., 2010a). SNB motoneurons were identified by their characteristic medial location in the L5-S1 spinal segment, and their large, multipolar somata (Fig. 6.1). Regardless of immunostaining density, the optical density of all identified SNB somata were measured (Stereo Investigator, MBF Bioscience, Inc.) as average luminosity per pixel (in 256 shades of gray, where black = 0 and white = 256) at a final magnification of 1480X under bright field illumination. To control for differences in

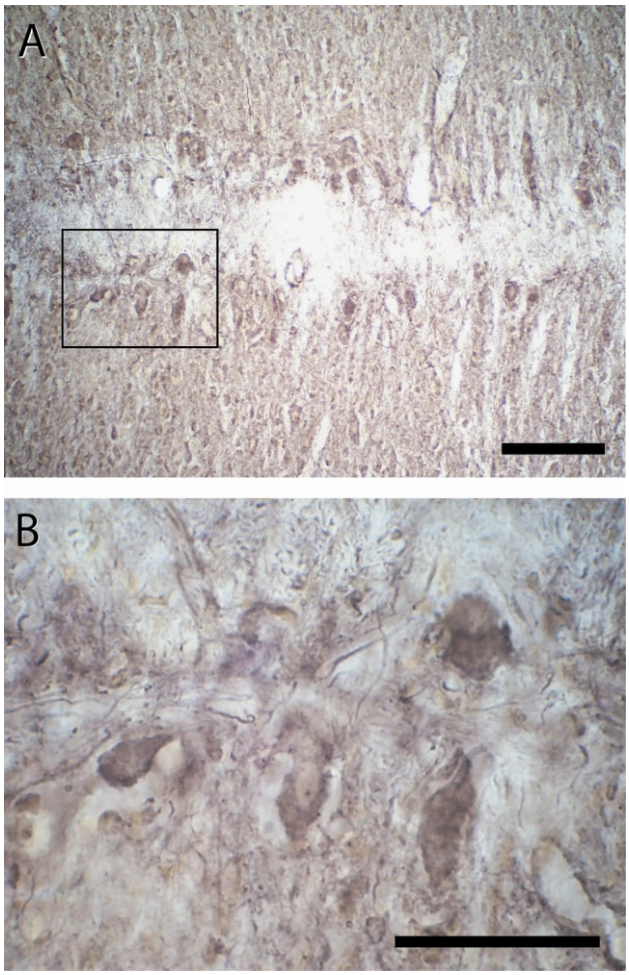

Figure 6.1. Digital light micrographs of horizontal sections of regions of the spinal cord containing BDNF immunolabeled SNB motoneurons from a gonadally intact male at P21. (A) Lower magnification illustrating the medial location of SNB motoneurons. Inset indicates higher magnification image of SNB motoneurons shown in (B). Scale bars = 100 μm.

immunostaining across sections and animals, optical density measures within each section were expressed relative to immunostaining in the adjacent white matter. BDNF immunostaining in white matter does not vary with hormonal condition using this immunolabeling protocol (Verhovshek and Sengelaub, 2013). Immunolabeled somata were categorized as intensely, moderately, or lightly labeled, according to their optical density values compared to mean optical density of BDNF immunolabeled somata in intact control males. This method of immunolabel quantification does not attempt to assess the actual amount of BDNF in motoneurons, but provides a reliable method for categorizing cells by immunostaining for subsequent analyses (Verhovshek and Sengelaub, 2010b).

RESULTS

The optical density of an average of 64.96 + 4.67 SNB motoneuron somata was assessed over an average of 3.08 ± 0.20 sections per animal. In normal males, the mean of the relative optical density of SNB motoneuron somata immunolabeled for BDNF was 0.80 with a standard deviation of 0.11. Intensely immunolabeled somata comprised 19.5% of the population, moderately immunolabeled somata comprised 60.4% of the population, and lightly immunolabeled somata comprised the remaining 20.1% of the population of SNB somata. BDNF immunolabeling was not significantly changed in response to hormonal condition [$F(4,38) = 1.75$, n.s.; Fig. 6.2]. In untreated castrates compared to normal males, there was a moderate, non-significant decrease in the proportion of intensely labeled cells (4.2%) while the proportion of moderately labeled SNB somata was similar (64.8%), and there was a non-significant increase (31%) in the proportion of lightly labeled cells [$F(1,14) = 1.53$, n.s.]. Castrates treated with estradiol at the BC muscle had proportions of intensely (13.4%), moderately (72.7%) and lightly-labeled (13.9%) somata that did not differ from those of castrated males [$F(1,18) = 2.22$, n.s.]. Castrates treated with estradiol at the interscapular region had proportions of intensely- (1.6%), moderately- (75.32%), and lightly-labeled (8%) somata that were comparable to those of untreated castrates [$F(1,14) = 3.07$, n.s.] and castrates treated with estradiol at the BC muscle

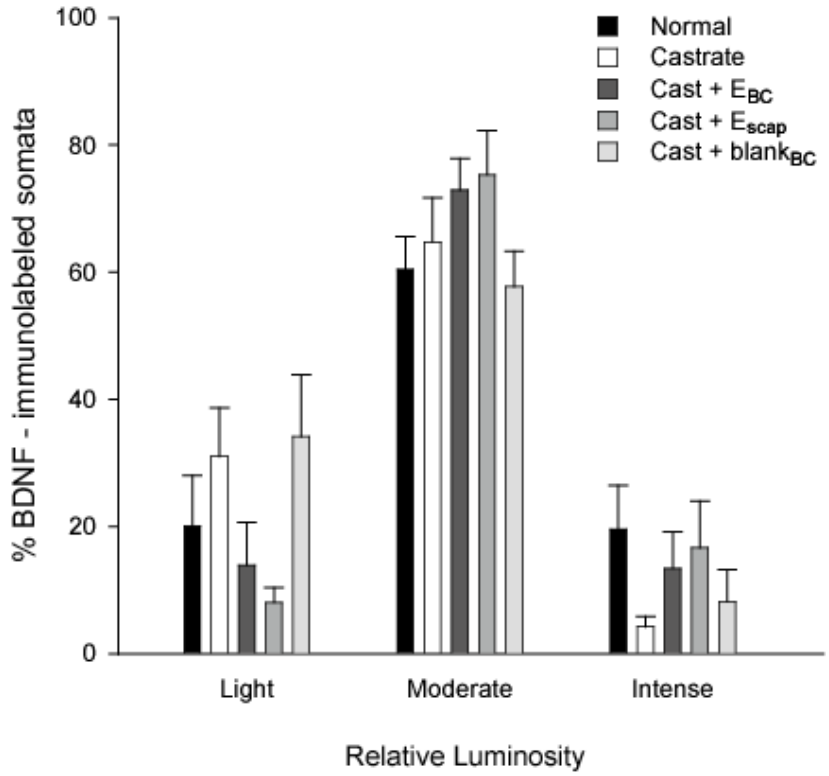

Figure 6.2. BDNF-immunolabeled SNB somata at P21 in normal males, untreated castrates (castrate), castrates treated with estradiol implants from P7-21 at the BC (castrate + E_{BC}), castrates treated with interscapular estradiol implants from P7-21 (castrate + E_{scap}), and castrates treated with a blank implant at the BC from P7-21 (cast + $blank_{BC}$). Percentages of lightly-, moderately, and intensely-immunolabeled SNB somata did not differ across groups.

[*F*(1,16) = 0.22, n.s.]. Castrates treated with blank implants at the BC muscle had a proportions of intensely (8.2%), moderately (54.7%), and lightly labeled (34.12%) somata that were similar to those of untreated castrates [*F*(1,16) = 0.29, n.s.].The mean cross-sectional area of SNB somata was not different across groups [*F*(4,19) = 0.49, n.s.].

Experiment 2

MATERIALS AND METHODS

BDNF ELISA. On P21, two weeks after hormonal manipulations, animals were euthanized by rapid decapitation. BC muscles were immediately removed, weighed, snap frozen on dry ice, wrapped in foil, transferred to polypropylene microcentrifuge tubes, and stored at -80°C. On the day of homogenization, 5 µl protease inhibitor cocktail (P8340; Sigma, St. Louis, MO, USA) was added to each ml of prepared homogenization buffer (100mM Tris HCl, pH 7, 2% BSA, 1M NaCl, 4mM EDTA, 2% Triton X-100, 0.1% sodium azide). Muscles were cut into small (~ 1 mm) pieces and placed in cold homogenization buffer (50 mg wet weight muscle per ml). Muscle was thoroughly homogenized using a rotor-stator homogenizer and homogenates were kept on ice until centrifugation (3200*g*, 30 min, 4°C). Resulting supernatants were transferred to polypropylene microcentrifuge tubes and stored at -80°C. On the day of the BDNF assay, frozen supernatants were thawed, briefly vortexed, and re-centrifuged (14000*g*, 30 min, 20°C) before analysis. Samples were diluted 1:4 in sample diluent and run in duplicate using a single commercial BDNF ELISA kit (CYT306, EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The following protocol parameters were used: overnight incubation was performed at 5°C with no shaking, mouse anti-BDNF incubation took place for 3 h on a shaker plate (200 rpm), and streptavidin-HRP incubation was performed with shaking (200 rpm). Following addition of the stop solution, optical density was read immediately at 450 nm (Epoch microplate spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA). Final BDNF concentrations were calculated using a linear regression model (Gen5 Data Analysis Software, BioTek) and data were expressed relative to the wet muscle mass (pg BDNF/mg tissue). Assay sensitivity was 7.8 pg/ml and intra-assay variation averaged 3.46% (range, 0.3 to 10.8). The assay was validated for parallelism by comparing a serial dilution of pooled muscle homogenate

to the BDNF standard curve (r^2 = 1.0). Recovery of known amounts of BDNF standard added to a pool of muscle homogenate was 114.3 ± 5.2% (y = 0.98x + 11.2; r^2 = 0.98). Because changes in BDNF concentration might be secondary to changes in overall protein levels in the muscle, we determined if castration and estradiol treatment affected total protein levels in the BC muscle. Total protein was measured in muscle homogenates using a microplate colorimetric assay (DC Protein Assay II, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, creating standards of 1.43, 0.715, 0.3575, and 0.17875 mg/ml for the curve. Samples were further diluted 1:25 in ultrapure water before analysis. As recommended by the manufacturer, standard curves were prepared in both the original sample matrix (Millipore BDNF sample diluent) and ultrapure water. Resulting absorbance values for each standard concentration were plotted using a linear regression model (Gen5) and the standard curves for sample diluent versus ultrapure water were compared to confirm the absence of assay interference by the sample diluent (r = 0.9). Total protein concentrations for each sample were then calculated using the standard curve prepared with sample diluent. Intra- assay CV averaged 3.9% (range, 0.5 to 9.09).

RESULTS

BDNF levels varied significantly across groups [F(4,23) = 4.65; Fig. 6.3]. Compared to intact control males (1.97 ± 0.14 pg/mg), BDNF levels were significantly greater in untreated castrates (2.85 ± 0.18 pg/mg; increased 45%), castrates treated with estradiol at the BC muscle (3.27 ± 0.28 pg/mg), or interscapular region (3.07 ± 0.22 pg/mg), and castrates with a blank implant at the BC muscle (2.62 ± 0.25 pg/mg; LSDs p < 0.05). These data demonstrate that castration results in an increase in BDNF in the BC muscle. BDNF levels in castrates treated with estradiol, regardless of treatment location, had BDNF levels that were no different than those of untreated castrates or castrates with a blank implant at the muscle, demonstrating that estradiol has no effect on BDNF levels in the SNB target muscle during the early postnatal period. There was no difference in total protein in the BC across groups [F (4,23) = 1.43; Fig. 6.4]. Because protein in the BC muscle was not affected by castration, as BDNF levels were, these data

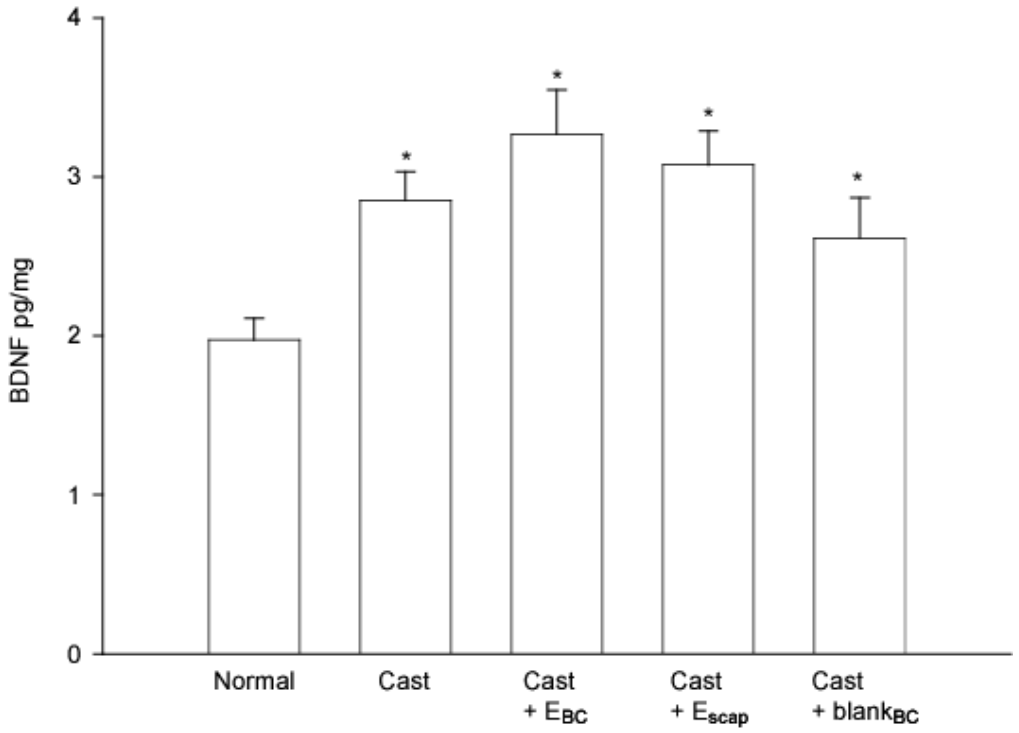

Figure 6.3. BDNF levels in the BC muscle at P21 in normal males, untreated castrates (castrate), castrates treated with estradiol implants from P7-21 at the BC (castrate + E_{BC}), castrates treated with interscapular estradiol implants from P7-21 (castrate + E_{scap}), and castrates treated with a blank implant at the BC from P7-21 (cast + $blank_{BC}$). * significantly different from normal males.

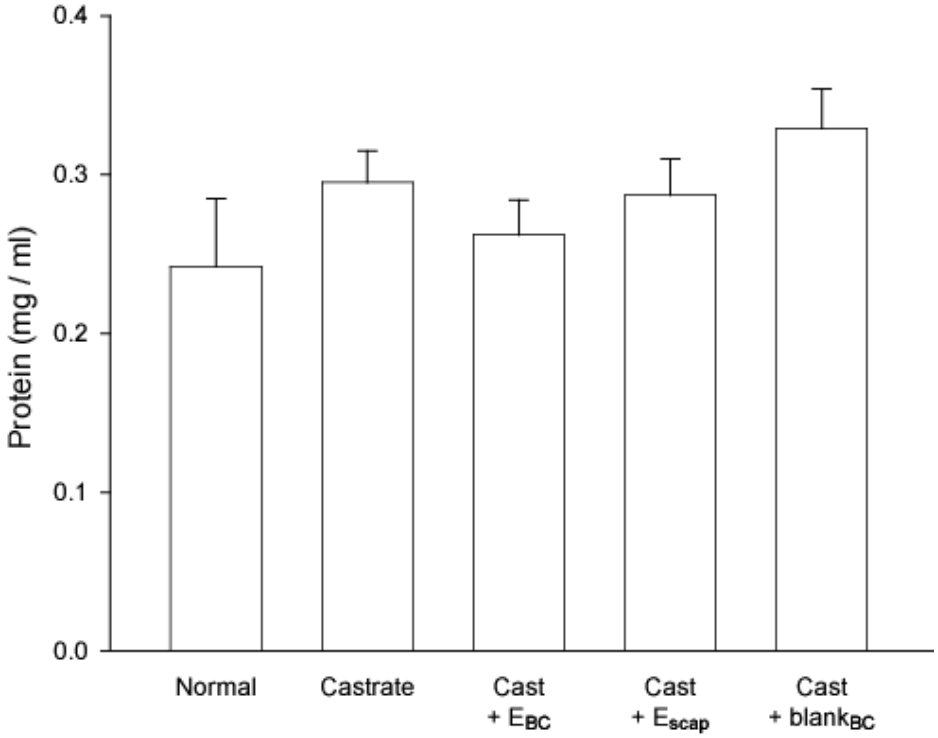

Figure 6.4. Protein levels in the BC muscle at P21 in normal males, untreated castrates (castrate), castrates treated with estradiol implants from P7-21 at the BC (castrate + E_{BC}), castrates treated with interscapular estradiol implants from P7-21 (castrate + E_{scap}), and castrates treated with a blank implant at the BC from P7-21 (cast + $blank_{BC}$).

demonstrate that the castration-induced change in BDNF is due to increases in BDNF itself, and not to changes in overall protein levels in the muscle.

DISCUSSION

In the experiments of this Chapter, I determined the effects of castration and estrogen treatment on BDNF levels in SNB motoneurons and their target muscles during the developmental period when estrogen-dependent SNB dendrite growth occurs. Results demonstrate that BDNF immunolabeling in SNB motoneurons does not change in response to castration or estradiol treatment, as the relative frequency of lightly, moderately, and intensely labeled SNB somata did not differ significantly across treatment groups. In the BC muscle, there was a significant increase in BDNF levels following castration, and neither a blank implant at the BC muscle or estradiol treatment (interscapular or at the BC) affected the castration-induced increase in muscle BDNF levels. Together, these data demonstrate that castration affects BDNF levels in the SNB target muscle, but not in motoneurons and that regardless of the castration effects on BDNF in the SNB neuromuscular system, estradiol treatment of castrates, whether interscapular or at the BC muscle, has no effect on BDNF immunolabeling in motoneurons, or BDNF protein in the BC muscle. This suggests that estrogen-dependent SNB dendrite growth does not occur through changes in BDNF in SNB motoneuron somata during the early postnatal period, as muscle and motoneuron BDNF did not change in response to estradiol treatment when estradiol is promoting dendrogenesis in SNB motoneurons.

BDNF immunolabeling

In this experiment, I found that in postnatal development the proportion of SNB somata lightly, moderately, and intensely immunolabeled for BDNF did not change significantly in response to castration or estradiol treatment (interscapular or at the BC muscle). These results are in contrast to previous results finding that in adulthood, castration results in a significant shift in BDNF immunolabeling in SNB somata: the percentage of intensely labeled somata decreases, with a concomitant increase in the percentage of somata lightly labeled for BDNF (Verhovshek et al., 2010a). Interestingly, there is a non-significant trend in the current data that follow the results

found in adulthood: castration moderately increases the proportion of lightly labeled somata while decreasing the proportion of SNB somata that are intensely labeled for BDNF. This shift in intensely to lightly immunolabeled somata follows the same pattern of results observed in adulthood after castration. Further, there is a non-significant trend in the current data that estradiol treatment at the BC restores BDNF immunolabeling to the levels observed in gonadally intact animals, suggesting that estradiol treatment at the SNB target muscle affects BDNF levels in SNB motoneurons, and that estrogen-dependent SNB dendrite growth could be mediated by changes in somal BDNF during the early postnatal period. Curiously, BDNF immunolabeling in castrates treated with interscapular estradiol is similar to that of castrates receiving estradiol treatment at the BC muscle. One explanation for these data is that the interscapular estradiol dose was too high, causing systemic elevations in estradiol that acted through the SNB target muscle. Previous data has demonstrated that gonadal hormone regulation of BDNF immunolabeling in SNB somata occurs through hormone action at the SNB target muscle (Verhovshek and Sengelaub, 2013), so excess estradiol from interscapular implants could act at the BC muscle to cause changes in BDNF immunolabeling in SNB somata that mimicked those found in castrates treated with estradiol at the BC muscle. However, interscapular implants containing an identical amount of estradiol do not alter SNB dendritic morphology in castrates (Nowacek and Sengelaub, 2006; Rudolph and Sengelaub, 2013a; Rudolph and Sengelaub, 2013b). Because the same estradiol interscapular treatment alters BDNF immunolabeling in SNB somata but has no effect on promoting SNB dendritic morphology, this change in somal BDNF cannot be responsible for any effects on SNB motoneuron dendrite growth. Therefore, the small but not significant alterations in BDNF immunolabeling observed in castrates treated with interscapular implants does not mediate estrogen-dependent dendrite growth, regardless of the site or mechanism of estrogenic regulation of BDNF in motoneurons. It is possible that BDNF immunolabeling in SNB motoneurons is more sensitive to estradiol than dendritic morphology during this time, and interscapular estradiol implants cause an increase in estradiol levels that are high enough to alter somal BDNF via the SNB target muscle, but not high enough to mediate SNB dendrite growth. However, BDNF immunolabeling in SNB motoneurons is comparable in

castrates treated with either interscapular estradiol or at the BC. If somal BDNF is mediated by estradiol at the BC muscle, it would be expected that estradiol treatment at the BC muscle would elicit more robust changes in BDNF in SNB motoneurons than estradiol treatment in the interscapular region. This further suggests that somal BDNF is not mediating the estrogen-dependent dendrite growth, as similar levels of BDNF immunolabeling are found in SNB somata in both muscle and interscapular estradiol treatment, but only muscle-localized estradiol has an effect on SNB dendrogenesis.

It is possible that the group differences in BDNF immunolabeling failed to reach significance due to small numbers of animals in each group (4-6). However, robust, significant differences in BDNF immunolabeling in adult SNB motoneurons have been observed with similar numbers of animals (5-6) in each group, and there have been many demonstrations of significant group differences in motoneuron morphology (e.g., Rudolph and Sengelaub, 2013a; Rudolph and Sengelaub, 2013b) and BDNF protein (e.g., current chapter) using similar group numbers. Based on these reasons, it is likely that the absence of significant group differences in BDNF immunolabeling in SNB motoneurons is a real effect, and not a statistical power issue.

Given that there appears to be no difference in BDNF immunolabeling in response to castration or estradiol treatment during development, these results stand in contrast to the findings in adulthood that, in general, castration decreases BDNF immunolabeling and testosterone treatment restores BDNF immunolabeling to gonadally intact levels (Verhovshek et al., 2010a). One explanation for the different pattern of results across developmental time points (P21 vs. adulthood) could be the time period between castration and assessment of BDNF immunolabeling. No change in BDNF in SNB somata was observed two weeks after castration in early development, but an overall decrease in somal BDNF was measured three weeks post-castration in adulthood. It is possible that castration-induced changes in somal BDNF occur, but that this change requires more than two weeks (e.g., three weeks) to be detected using our immunohistochemical technique. However, BDNF mRNA is altered in response to castration after only two weeks in adulthood (Ottem et al., 2007), suggesting that changes in somal BDNF levels can occur within a shorter period of time. It is important to note that changes in BDNF

observed within two weeks of castration in adulthood occurred with BDNF mRNA, not BDNF protein, and it is important to note that alterations in mRNA versus protein do not always follow the same pattern or time course of changes (Solum and Handa, 2002), and we did not assess changes in BDNF mRNA. Further, we have observed robust changes in BDNF levels in other components of the SNB neuromuscular system (i.e., BC muscle) during this development period two weeks post-castration. Another possible explanation for the different pattern of changes observed in somal BDNF in the SNB after castration is that developmental and adult castration result in significantly different changes in gonadal hormones, depending on whether the procedure is performed during development or in adulthood. At P7, serum testosterone levels are significantly lower (~0.6 ng/ml) than they are in adulthood (~3.5 ng/ml; Döhler and Wuttke, 1975; Walker et al., 2012), so early castration results in a significantly smaller change in testosterone levels compared to adult castration. If BDNF levels are sensitive to the magnitude of change in serum testosterone, it is possible that changes in BDNF would only occur when changes in circulating testosterone are large enough to elicit changes in BDNF (e.g., in adulthood).

BDNF ELISA

In this study, I found that BDNF levels in the BC muscle increase by 45% two weeks following castration. These results replicate the findings from adulthood that demonstrate castration-induced increases in BC BDNF. However, estradiol treatment (regardless of treatment location) had no effect on the castration-induced increases in muscle BDNF. The failure of estradiol treatment to attenuate this increase in BDNF suggests that muscle BDNF, similar to somal BDNF, is not regulated by estrogens. In adulthood, testosterone treatment fully prevents the castration-induced increase in muscle BDNF, demonstrating that peripheral BDNF levels are androgen-regulated in the SNB system. We did not assess the ability of androgens to alter BDNF levels in the BC during development, so it is possible that BDNF levels in the BC are regulated by androgens, not estrogens during development.

While early castration does not significantly alter BDNF immunolabeling in SNB somata, BDNF protein in the SNB target muscle increases significantly in response to castration on P7,

suggesting that early castration differentially alters BDNF in the SNB target muscle, but not in SNB somata. One explanation for this could be a difference in androgen sensitivity of the SNB target muscle compared to SNB somata. Neonatally, ARs are present in the BC muscle, but not in SNB motoneurons (Fishman et al., 1990). Further, at P7, AR immunoreactivity in the SNB target muscle is not different from its adult levels. In contrast, on P7, AR immunolabeling in SNB motoneurons is significantly lower than its adult levels (Jordan et al., 1997). Given the relationship between AR density and androgen sensitivity (e.g., Huguenard et al., 2011), these data suggest that the BC muscle is more sensitive to androgens than SNB motoneurons on P7, when castrations were performed. If BDNF levels in the SNB system are uniquely androgen sensitive during early development, the differential androgen sensitivity of the SNB target muscle compared to SNB somata explains why BDNF levels are altered by castration in the BC, but not somata. However, in adulthood, the site of action for androgenic regulation of BDNF levels in SNB somata is the target muscle itself (Verhovshek and Sengelaub, 2013), suggesting that the AR density in the SNB target muscle, not the motoneurons, is the critical aspect for the regulation of BDNF in both central and peripheral components of the SNB system. While it is unlikely that the site of action for regulation of BDNF in SNB motoneurons differs from development to adulthood, experiments to address this site of action in development should be performed.

Another explanation for the potentially conflicting results could be a reflection of the differential sensitivity of the methods used to measure BDNF in somata versus muscle. The immunolabeling technique used to assess BDNF in SNB motoneurons is semi-quantitative and reports optical density as a proxy for BDNF levels, whereas BDNF levels in the SNB target muscle were measured by ELISA, and levels of BDNF are reported as pg/mg, arguably, a much more sensitive measure of BDNF than immunolabeling optical density in SNB somata. In adulthood, the castration-induced change in circulating testosterone levels is large, potentially eliciting a greater change in BDNF in the SNB neuromuscular system that is detectable by both immunolabeling and ELISA. However, the much lower levels of circulating testosterone at P7, results in a smaller change in androgens following castration on P7, resulting in smaller changes in BDNF, which are detectable only by the more sensitive ELISA technique. It is possible that

castration-induced increases in muscle BDNF protein could be the result of a general increase in peripheral protein production following castration. However, there was no difference in overall protein levels in the BC group following castration, demonstrating that the changes observed in BDNF levels were due to changes in BDNF protein alone, and not to a general increase in BC muscle protein.

Regardless of the reason for a differential regulation of BDNF in the peripheral and central components of the SNB system, it remains that BDNF levels increased in the SNB target muscle two weeks after early castration. A post-castration increase in BDNF in the muscle could be explained by a decrease in retrograde transport of muscle-produced BDNF to SNB motoneuron. The castration-induced increase in muscle BDNF could reflect a decrease in retrograde transport of BDNF from the BC/LA to SNB motoneurons or an increase in peripheral BDNF production in the absence of gonadal hormones. Additionally, the site of action of BDNF regulation of SNB motoneuron morphology is unknown. It is possible that muscle BDNF directly alters dendritic morphology via retrograde transport. Alternatively, a change in BDNF in the muscle could be the initial step in a molecular cascade that ultimately alters the cellular composition of SNB motoneurons, and dendritic morphology could be regulated by molecular changes in the motoneurons. Future studies should determine whether changes in dendritic morphology secondary to changes in muscle BDNF are controlled by muscle- or motoneuron-localized events, and if they are directly regulated by BDNF or by secondary molecular pathways.

Regardless of the mechanism, the castration-induced increase in muscle BDNF coincides with a failure of SNB dendrites to grow, suggesting that BDNF has an inhibitory role on SNB dendrites during early development. In adulthood, blockade of BDNF using a trkB IgG alters SNB dendritic morphology and soma size (Verhovshek and Sengelaub, 2010), but this method of altering BDNF signaling has not been used developmentally to determine if there is a role for BDNF in the growth of SNB dendrites during the early postnatal period. Given that BDNF is involved in the masculinization of SNB cell number (Xu et al., 2001), and changes in gonadal hormones during the first few postnatal weeks alters BDNF at the site of action for SNB dendrite growth (current chapter), future studies should determine if BDNF affects SNB motoneuron

morphology during early development as it does in adulthood by blocking BDNF signaling with trkB IgG.

CONCLUSIONS

In this chapter, I have demonstrated that early castration results in an increase in BDNF in the SNB target muscle, but no significant change in BDNF immunolabeling in SNB somata. In both cases, estradiol treatment does not alter BDNF levels, suggesting that BDNF in peripheral and central components of the SNB system is not regulated by estrogens during the early postnatal period. These data rule out a role for BDNF in the molecular mechanisms of estrogen-dependent SNB dendrogenesis, and suggest that another molecular pathway is involved in mediating dendrite growth during this developmentally restricted critical period. This is in contrast to the adult SNB system, in which androgens maintain SNB morphology through the modulation of BDNF. Together, these data suggest that different molecular pathways underlie the estrogenic versus androgenic components of SNB dendritic growth and maintenance, respectively. The molecular mechanisms of estrogen-dependent dendrogenesis remain to be determined, but candidate molecular pathways include estradiol regulation of insulin-like growth factor-I (Arevalo et al., 2012), or via a Src/Ras/ERK pathway (Miñano et al., 2008). IGF-I and ERs colocalize in many nervous system structures (e.g., Garcia-Segura et al., 2010), and IGF-I has been shown to promote neuritogenesis in an ERα-dependent manner (e.g., Ma et al., 1994). IGF-I and estradiol interact developmentally, and these interactions have been shown regulate features of neural development, including the differentiation of sexually dimorphic structures critical for neuroendocrine control of reproductive processes (Carrer and Cambiasso, 2002).

It is possible that androgen- and estrogen-dependent dendrite growth during the early postnatal period are both mediated by a non-BDNF mechanism. This would suggest that the mechanisms of dendritic growth are different than the mechanisms of maintenance of SNB dendritic morphology, as we know BDNF is involved in the regulation of SNB dendritic lengths in adulthood. Further, this would indicate that molecular mechanisms of SNB dendrite growth are not hormone-specific, but temporal-specific, as early dendrite growth (either estrogen- or

androgen-dependent) could be mediated by a non-BDNF mechanism, whereas SNB dendritic maintenance is mediated by BDNF. Experiments should be performed to determine if SNB dendrogenesis during early development occurs through one molecular pathway, regardless of which gonadal hormone is mediating the dendrite growth. If the molecular pathway mediating SNB dendrogenesis is not conserved across gonadal hormones during early development, it remains possible that androgen regulation of SNB dendrites throughout the lifespan is mediated by BDNF, and estrogen regulation of SNB dendritic morphology occurs through a distinctly separate molecular pathway. To answer these questions, future studies should assess androgenic regulation of BDNF during the early postnatal period in SNB motoneurons and their target muscles, and determine the effects of trkB IgG treatment on SNB motoneuron morphology during the early postnatal. Additionally, studies should assess the molecular pathways (e.g., IGF-I) mediating estrogen-dependent dendrite growth during early development.

CHAPTER 7: General Discussion

This dissertation has focused on identifying the mechanisms regulating the critical period for estrogen-dependent dendrite growth in the SNB. I have demonstrated that estrogen-dependent SNB dendrite growth occurs coincident with ERα expression in extra-muscle fiber cells of the SNB target muscle, and restricting estradiol treatment to the BC muscle during the period of maximal extra-fiber ERα expression results in full estrogen-dependent masculinization of SNB dendrites (Rudolph and Sengelaub, 2013a; Chapter 2). In Chapter 3 I showed that ERα-expressing extra-muscle fiber cells are regulated by early gonadal hormones, as castration results in an upregulation of ERα in the SNB target muscle (Rudolph and Sengelaub, 2013b). Further, brief estradiol treatment during the heightened ERα expression in the SNB target muscle results in rapid, robust dendritic growth, suggesting that extra-muscle fiber ERα density determines the estrogen sensitivity of SNB dendrites during early development. Muscle ERα appears to have a role in determining the temporal limits of the critical period for estrogen-dependent dendrogenesis as well. In Chapter 4 I demonstrated that castration-induced upregulation of extra-fiber ERα in the BC permits estrogen sensitivity of SNB dendrites outside the typical critical period for dendritic growth in response to estrogens. This was the first demonstration that SNB morphology can respond to estradiol treatment outside of the early postnatal period. The first 3 experimental chapters have solidified the importance of extra-muscle fiber ERα in the SNB target muscle in estrogen-dependent SNB dendrite growth, both within and outside of the early postnatal period. Chapter 5 ruled out satellite cells as the ERα-expressing extra-muscle fiber cells that appear to be the site of action for estrogen-dependent SNB dendrite growth. In Chapter 6, I investigated the potential role of BDNF in estrogen-dependent dendrite growth, assessing BDNF in SNB motoneurons and their target muscles after castration and estradiol treatment. The results from these experiments suggest that while peripheral BDNF is sensitive to castration, central BDNF is not. Most importantly for this thesis, BDNF in either component of the SNB neuromuscular system is insensitive to estradiol treatment, indicating that BDNF does not mediate estrogen-dependent SNB dendrite growth.

While this dissertation has answered many questions about the mechanisms of SNB dendrite growth in response to estrogens, a number of details about how estrogens promote SNB

dendrite growth remain unknown. One of the most striking findings from this work was discovering that estrogen-dependent SNB dendrite growth could occur outside the early postnatal period. Previously, SNB dendritic morphology was thought to be entirely estrogen-insensitive after P28, but I found that castration-induced maintenance of extra-fiber ERα in the BC permits dendrogenesis in response to estradiol treatment through P49. What remains unclear is the duration of estrogen responsiveness of SNB dendrites after early castration. We know that ERα is upregulated at P21 following gonadectomy on P7, and that treatment with estradiol from P28 to P49 results in typical dendritic lengths, but the time course and permanence of this ERα are unknown. Previous work performed on non-sexually dimorphic rodent skeletal muscle has shown that castration-induced upregulation of muscle ERα occurs within 3 weeks, and as little as 48 hours post-castration (Baltgavis et al., 2010). The only post-castration time point used in the current work for measurements of muscle ERα levels in the BC was 2 weeks, but it is likely that the ERα upregulation occurred before this time point. Given that during typical development, significant differences in BC extra-fiber ERα occur within 1 week, (the developmental downregulation of ERα from P14 to P21; Rudolph and Sengelaub, 2013a; Chapter 2), and in other skeletal muscles, ERα is upregulated 48 hours after castration (Baltgavis et al., 2010), it is likely that the increase in extra-fiber ERα following castration occurs earlier than 2 weeks. Future studies should determine the time course of castration-induced changes in extra-muscle fiber ERα of the BC muscle, both during typical development (e.g., time points within the 1-week intervals from P7 to P28 when the developmental downregulation occurs) and following castration.

In addition to determining the time course of developmental downregulation of extra-muscle fiber ERα, the duration of extra-muscle fiber ERα upregulation following castration should be assessed. In the current experiments, ERα was upregulated at P21 following castration on P7, and treatment with estradiol from P21 to P28 or P28 to P49 resulted in dendritic growth. This suggests that the castration-induced upregulation is stable at the minimum from P21 to P28, because estradiol treatment beginning at P28 results in SNB dendrite growth by P49. It is also possible that once upregulated via early castration, extra-muscle fiber ERα levels in the SNB

target muscle could remain stable indefinitely, creating an SNB system that is permanently estradiol sensitive. Similar to understanding the duration of castration-induced ERα upregulation, future studies should investigate how this enhanced ERα expression responds to exogenous estradiol treatment. It has been suggested that the typical downregulation of extra-muscle fiber ERα occurs due to high postnatal estradiol levels (Rudolph and Sengelaub, 2013a; Rudolph and Sengelaub, 2013b), and it is possible that exogenous estradiol treatment affects muscle ERα in a similar manner. If estradiol treatment in castrates downregulates BC extra-muscle fiber ERα, the time course of this downregulation should be assessed to understand when during the treatment period ERα levels are reduced to a point where SNB dendrites are no longer able to respond to estradiol treatment with dendritic growth. In P7 castrates, estradiol treatment from P21 to P28 and P28 to P49 results in robust dendritic growth by P28 and P49, respectively, but it is not known when in these treatment periods dendritic growth occurs. It is possible that SNB dendrites of P7 castrates respond on the first day of estradiol treatment with rapid, robust dendritic growth, and extra-muscle fiber ERα responds to this estradiol treatment by downregulating its receptor. In this scenario, the brief estradiol-ERα interaction at the muscle is sufficient to promote dendritic growth that persists through P28 and P49. It is also possible that the castration-induced ERα in BC extra-muscle fiber cells is stable, and estradiol treatment (either from P21 to P28 or P28 to P49) promotes a steady rate of dendritic growth throughout the estradiol treatment period. The time course of estradiol-dependent dendritic growth in P7 castrates treated with estradiol both during the early postnatal period and outside of this period should be assessed.

Another unanswered question that remains concerns the molecular mechanisms mediating estrogen-dependent SNB dendrite growth. In Chapter 6, I concluded that BDNF is not involved in estrogen-mediated dendrogenesis during the early postnatal period, and suggested that estrogens could be regulating other molecular messengers (e.g., IGF-I) to modulate dendritic morphology. These examples involve the classical actions of estradiol: receptor binding followed by translocation to the cell nucleus where the receptor-hormone complex acts as a transcription factor to regulate gene expression. However, recent advances in the understanding of estrogenic modulation of the morphology of neural structures that control sexually dimorphic behaviors

suggest that ERα could direct changes in cellular morphology via its actions at the cell surface (reviewed in Micevych and Christensen, 2012). In the hypothalamus, neurons in the arcuate nucleus that regulate lordosis undergo rapid, significant changes in dendritic spine morphology in response to estrogens acting through membrane receptors (Christensen et al., 2011). The membrane-localized effects of estradiol often require ER interaction with metabotropic glutamate receptors (mGluRs; reviewed in Boulware and Mermelstein, 2009), and ER-mGluR interactions have been shown to alter dendritic morphology resulting in changes in sexual behavior, including lordosis (Christensen et al., 2011). Importantly, estradiol effects on skeletal muscle have been shown to be mediated by membrane ERα (Galluzzo et al., 2009; Hatae et al., 2009), suggesting that estradiol action at ERα-expressing extra-muscle fiber cells could be occurring at the cell surface. Future studies should assess the potential for membrane-bound ERα to regulate estrogen-dependent SNB dendrite growth during the early postnatal period.

In adulthood, the mechanism sustaining SNB dendritic lengths is known: BDNF mediates the androgen-dependent maintenance of SNB dendritic morphology, but it is unclear what the mechanism is that mediates the androgen component of dendritic growth during development. Given that BDNF blockade perinatally affects the androgen-dependent regulation of SNB cell number (Xu et al., 2001), there is a clear role for BDNF in SNB motoneuron development. BDNF blockade in adulthood affects not only SNB dendritic lengths, but soma size as well (Verhovshek and Sengelaub, 2010) demonstrating that the androgen-dependent effects of BDNF on SNB motoneurons occurs both developmentally and in adulthood. Therefore, it is likely that BDNF plays a role in SNB dendrite growth during development, potentially through androgen-mediated pathways. Future studies should determine if BDNF mediates the androgen component of SNB dendrite growth during the early postnatal period to address the possibility that the androgen-dependent growth and retraction of SNB dendrites occurs through a conserved molecular pathway.

The convergence of two molecular pathways mediating dendritic growth, one via estrogens and one via androgens, could explain the pattern of dendritic growth (P7-P28; Goldstein and Sengelaub, 1994) and retraction (P28-P49; Goldstein et al., 1990) that occurs in

the SNB during development. If androgens and estrogens both promote dendrite growth (via distinct pathways) through P28, this could explain the robust dendritic growth that occurs from P7 to P28. At P28, due to the downregulation of extra-muscle fiber ERα, estrogens lose their effectiveness in promoting dendrogenesis. When the SNB becomes insensitive to estrogens, the remaining effect of gonadal hormones on dendrogenesis is androgenic, and given the low, prepubertal levels of androgen, dendrites retract to their adult lengths (Goldstein et al., 1990). This dual process model of SNB dendritic growth and maintenance explains why SNB dendritic growth is only partial when either estradiol or dihydrotestosterone alone is supplied during development (Goldstein and Sengelaub, 1994), but when combined fully support SNB dendrite growth through P28 (Burke et al., 1997). Additionally, this model accounts for why SNB dendrite growth is incomplete with fadrozole treatment: this aromatase blockade eliminates the estradiol component of SNB dendrite growth (Burke et al., 1999). These data support the idea of a dual process model in which androgens and estrogens act through different molecular mechanisms to regulate SNB dendrogenesis. To fully understand how androgens and estrogens mediate this SNB dendritic growth during development, the molecular mechanisms of androgen- and estrogen-dependent SNB dendrite growth must be determined.

Estrogen-dependent SNB dendrite growth is mediated by extra-fiber ERα in the SNB target muscle, and SNB dendrites become insensitive to estrogens when ERα is downregulated in the BC muscle. The mechanism of developmental downregulation of extra-muscle fiber ERα is unknown, but gonadal hormones are involved in this process, as early castration prevents the typical downregulation of ERα in the muscle. Previous work has demonstrated that estradiol can downregulate ERα, in skeletal muscle (Baltgavis et al., 2010) and in a variety of structures across the neuraxis (e.g., Shughrue et al., 1992; Sohrabji et al., 1994). Estradiol levels are very high during the early postnatal period (Döhler and Wuttke, 1975), suggesting that estradiol-induced downregulation of ERα in the BC is possible. More recent findings indicate that this estradiol-dependent regulation of ERα could be occurring through changes in DNA methylation patterns (reviewed in Imamura, 2011). Indeed, ERα promoter regions have been shown to be downregulated by estradiol (Saceda et al., 1988), potentially via changes in DNA methylation.

Once established, DNA methylation patterns can be quite stable, allowing for estradiol-dependent changes in methylation of promoter regions of ERα to maintain ERα expression at low levels after downregulation has occurred during the early postnatal period. Removal of estradiol via castration would eliminate any estradiol-dependent changes in DNA methylation, preventing the downregulation of muscle ERα and resulting in estrogen sensitive SNB dendrites outside the early postnatal period. This provides a mechanism for how early gonadal hormone exposure establishes the transient pattern of estrogen sensitivity observed in the SNB system. Future studies should determine if the developmental downregulation of extra-muscle fiber ERα occurs through estrogen-dependent changes in DNA methylation patterns. The stability of these methylation patterns should be assessed in order to understand the potential permanence of the castration-induced upregulation of ERα. In conclusion, this thesis has examined many mechanisms of estrogen-dependent SNB dendrite growth, including the role of extra-muscle fiber ERα, the sensitivity of extra-fiber ERα to early gonadal hormone exposure and the relationship of changes in ERα to estrogen sensitivity of SNB dendrites, and the potential role of BDNF in estrogen-dependent dendrite growth. This research has provided valuable insights into the understanding of estrogen-mediated differentiation of sexually dimorphic neuromuscular system, and future studies should build upon these results to fully understand not only the complete set of mechanisms of estrogen-dependent dendrite growth, but how androgens and estrogens interact developmentally to direct the growth and retraction of SNB dendrites.

REFERENCES

Arevalo MA, Ruiz-Palmero I, Scerbo MJ, Acaz-Fonseca E, Cambiasso MJ, Garcia-Segura LM. 2012. Molecular mechanisms involved in the regulation of neuritogenesis by estradiol: Recent advances. J Steroid Biochem Mol Biol. 131(1-2):52-6.

Baltgavis KA, Greising SM, Warren GL, Lowe DA. 2010. Estrogen regulates estrogen receptors and antioxidant gene expression in mouse skeletal muscle. PLoS One 5(4):e10164.

Banie L, Lin G, Ning H, Wang G, Lue T, Lin C. 2008. Effects of estrogen, raloxifene and levormeloxifene on alpha1A-adrenergic receptor expression. J Urol 180:2241-2246.

Becchis M, Sullivan PM, Ordronneau P, Petrusz P, Joseph DR. 1996. Distribution of immunoreactive androgen-binding protein/sex hormone-binding globulin in tissues of the fetal rat. Steroids. 61(7):392-400.

Boulware M, Mermelstein, PG. 2009 Membrane estrogen receptors activate metabotropic glutamate receptors to influence nervous system physiology. Steroids. 74(7):608-13.

Breedlove SM, Arnold AP. 1980. Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord. Science 210:564-566.

Breedlove SM, Arnold AP. 1981. Sexually dimorphic motor nucleus in the rat lumbar spinal cord: response to adult hormone manipulation, absence in androgen-insensitive rats. Brain Res 225:297-307.

Breedlove SM, Jacobson CD, Gorski RA, Arnold AP. 1982. Masculinization of the female rat spinal cord following a single neonatal injection of testosterone propionate but not estradiol benzoate. Brain Res 237:173-181.

Breedlove SM, Arnold AP. 1983. Hormonal control of a developing neuromuscular system. I. Complete Demasculinization of the male rat spinal nucleus of the bulbocavernosus using the anti-androgen flutamide. J Neurosci 3:417-423.

Breedlove SM, Arnold AP. 1983. Hormonal control of a developing neuromuscular system. II. Sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus. J Neurosci 3:424-432.

Burke KA, Widows MR, Sengelaub DR. 1997. Synergistic effects of testosterone metabolites on the development of motoneuron morphology in a sexually dimorphic rat spinal nucleus. J Neurobiol 33:1-10.

Burke KA, Kuwajima M, Sengelaub DR. 1999. Aromatase inhibition reduces dendritic growth in a sexually dimorphic rat spinal nucleus. J Neurobiol 38:301-312.

Carrer HF, Cambiasso MJ. 2002. Sexual differentiation of the brain: genes, estrogen, and neurotrophic factors. Cell Mol Neurobiol. 22(5-6):479-500.

Christensen A, Dewing P, Micevych P. 2012. Membrane-initiated estradiol signaling induces spinogenesis required for female sexual receptivity J Neurosci. 31(48):17583-9.

Čihák R, Gutmann E, Hanzlíková V. 1970. Involution and hormone-induced persistence of the M. sphincter (levator) ani in female rats. J Anat 106:93-110.

Coggeshall R. 1992. A consideration of neural counting methods. Trends Neurosci 15:9-13.

Couse JK, Lindzey J, Grandien K, Gustafsson JA, Korach KS. 1997. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology 138: 4613-21.

Döhler KD, Wuttke W. 1975. Changes with age in levels of serum gonadotropins, prolactin and gonadal steroids in prepubertal male and female rats. Endocrinology 97(4):898-907.

Dube JY, Lesage R, Tremblay RR. 1976. Androgen and estrogen binding in rat skeletal and perineal muscles. Can J Biochem 54:50-55.

Fargo KN, Foster AM, Harty MW, Sengelaub DR. 2003. Estrogen alters excitability but not morphology of a sexually dimorphic neuromuscular system in adult rats. J Neurobiol 56:66-77.

Fishman RB, Chism L, Firestone GL, Breedlove SM. 1990. Evidence for androgen receptors in sexually dimorphic perineal muscles of neonatal male rats. Absence of androgen accumulation by the perineal motoneurons. J Neurobiol. 21(5):694-704.

Floryk D, Kurosaka S, Tanimoto R, Yang G, Goltsov A, Park S, Thompson TC. 2011. Castration-induced changes in mouse epididymal white adipose tissue. Mol Cell Endocrinol. 345(1-2):58-67.

Freeman L, Watson N, Breedlove SM. 1996. Androgen spares androgen-insensitive motoneurons from apoptosis in the spinal nucleus of the bulbocavernosus in rats. Horm Behav 30:424-433.

Galluzzo P, Rastelli C, Bulzomi P, Acconcia F, Pallottini V, Marino M. 2009. 17 beta-estradiol regulates the first steps of skeletal muscle cell differentiation via ER-alpha-mediated signals. Am J Physiol Cell Physiol. 297(5):C1249-62.

Garcia-Segura LM, Arévalo MA, Azcoitia I. 2000. Interactions of estradiol and insulin-like growth factor-I signalling in the nervous system: new advances. Prog Brain Res. 181:251-72.

Goldstein LA, Kurz EM, Sengelaub DR. 1990. Androgen regulation of dendritic growth and retraction in the development of a sexually dimorphic spinal nucleus. J Neurosci 10:935-946.

Goldstein LA, Sengelaub DR. 1990. Hormonal control of neuron number in sexually dimorphic spinal nuclei of the rat. IV. Masculinization of the spinal nucleus of the bulbocavernosus with testosterone metabolites. J Neurobiol 21:719-730.

Goldstein LA, Sengelaub DR. 1992. Timing and duration of dihydrotestosterone treatment affect the development of motoneuron number and morphology in a sexually dimorphic rat spinal nucleus. J Comp Neurol 326:147-157.

Goldstein LA, Kurz EM, Kalkbrenner AE, Sengelaub DR. 1993. Changes in dendritic morphology of rat spinal motoneurons during development and after unilateral target deletion. Dev Brain Res 73:151-163.

Goldstein LA, Sengelaub DR. 1994. Differential effects of dihydrotestosterone and estrogen on the development of motoneuron morphology in a sexually dimorphic rat spinal nucleus. J Neurobiol 25:878-892.

Goldstein LA, Mills AC, Sengelaub DR. 1996. Motoneuron development after deafferentation .1. Dorsal rhizotomy does not alter growth in the spinal nucleus of the bulbocavernosus (SNB). Dev Brain Res 91:11-19.

Hart BL, Melese-D'Hospital PY. 1983. Penile mechanisms and the role of the striated penile muscles in penile reflexes. Physiol Behav 31:807-813.

Hatae J, Takami N, Lin H, Honda A, Inoue R. 2009. 17 beta-Estradiol-induced enhancement of estrogen receptor biosynthesis via MAPK pathway in mouse skeletal muscle myoblasts. J Physiol Sci. 59(3):181-90.

Hayes KJ. 1965. So-called levator ani of rat. Acta Endocrinol (Copenh) 48:337-347.

Hays TC, Goldstein LA, Mills AC, Sengelaub DR. 1996. Motoneuron development after deafferentation .2. Dorsal rhizotomy does not block estrogen-supported growth in the dorsolateral nucleus (DLN). Dev Brain Res 91:20-28.

Hebbeler SL, Verhovshek T, Sengelaub DR. 2002. N-methyl-D-aspartate receptor blockade inhibits estrogenic support of dendritic growth in a sexually dintorphic rat spinal nucleus. J Comp Neurol 451:142-152.

Hebbeler SL, Sengelaub DR. 2003. Development of a sexually dimorphic neuromuscular system in male rats after spinal transection: Morphologic changes and implications for estrogen sites of action. J Comp Neurol 467:80-96.

Huguenard A, Fernando SM, Monks DA, Sengelaub DR. 2011. Overexpression of androgen receptor in target musculature confers androgen sensitiviry to motoneuron dendrites. Endocrinology 152:639-650.

Imamura, T. 2011. Epigenetic setting for long-term expression of estrogen receptor α and androgen receptor in cells. Horm Behav. 59: 345-352.

Johansen JA, Breedlove SM, Jordan CL. 2007. Androgen receptor expression in the levator ani muscle of male mice. J Neuroendocrinol. 19(10):823-6.

Jordan CL, Watamura S, Arnold AP. 1995. Androgenic, not estrogenic, steroids alter neuromuscular synapse elimination in the rat levator ani. Brain Res. 84(2):215-24.

Jordan CL, Letinsky MS, Arnold AP. 1989a. The role of gonadal hormones in neuromuscular synapse elimination in rats. I. Androgen delays the loss of multiple innervation in the levator ani muscle. J Neurosci 9:229-238.

Jordan CL, Letinsky MS, Arnold AP. 1989b. The role of gonadal hormones in neuromuscular synapse elimination in rats. II. Multiple innervation persists in the adult levator ani muscle after juvenile androgen treatment. J Neurosci 9:239-247.

Jordan CL, Padgett B, Hershey J, Prins G, Arnold A. 1997. Ontogeny of androgen receptor immunoreactivity in lumbar motoneurons and in the sexually dimorphic levator ani muscle of male rats. J Comp Neurol. 379(1):88-98.

Jordan CL, Williams TJ. 2001. Testosterone regulates terminal Schwann cell number and junctional size during developmental synapse elimination. Dev Neurosci. 23(6):441-51.

Kahn SM, Li YH, Hryb DJ, Nakhla AM, Romas NA, Cheong J, Rosner W. 2008. Sex hormone-binding globulin influences gene expression of LNCaP and MCF-7 cells in response to androgen and estrogen treatment. Adv Exp Med Biol.;617:557-64.

Krizan-Agbas D, Pedchenko T, Hasan W, Smith PG. 2003. Oestrogen regulates sympathetic neurite outgrowth by modulating brain derived neurotrophic factor synthesis and release by the rodent uterus. Eur J Neurosci 18:2760-2768.

Kurz EM, Sengelaub DR, Arnold AP. 1986. Androgens regulate the dendritic length of mammalian motoneurons in adulthood. Science 232:395-398.

Kurz EM, Bowers CA, Sengelaub DR. 1990. Morphology of rat spinal motoneurons with normal and hormonally altered specificity. J Comp Neurol 292:638-650.

Kurz EM, Brewer RG, Sengelaub DR. 1991. Hormonally mediated plasticity of motoneuron morphology in the adult rat spinal cord: a cholera toxin-HRP study. J Neurobiol 22:976-988.Lee et al., 1989

Leslie M, Forger NG, Breedlove SM. 1991. Does androgen affect axonal transport of cholera toxin HRP in spinal motoneurons? Neurosci Lett 126:499-514.

Love FM, Thompson WJ. 1998. Schwann cells proliferate at rat neuromuscular junctions during development and regeneration. J Neurosci. 18(22):9376-85.

Lubischer JL, Bebinger DM. 1999. Regulation of terminal Schwann cell number at the adult neuromuscular junction. J Neurosci. 19(24):RC46.

Ma ZQ, Santagati S, Patrone C, Pollio G, Vegeto E, Maggi A. 1994. Insulin-like growth factors activate estrogen receptor to control the growth and differentiation of the human neuroblastoma cell line SK-ER3. Mol Endocrinol. 8(7):910-8.

McKenna KE, Nadelhaft I. 1986. The organization of the pudendal nerve in the male and female rat. J Comp Neurol 248:532-549.

Mesulam M. 1982. Tracing neural connections with horseradish peroxidase.: Chichester: Wiley. 268 pp.

Micevych P, Christensen A. 2012. Membrane-initiated estradiol actions mediate structural plasticity and reproduction. Front Neuroendocrinol. 33(4):331-41.

Milanesi L, Russo de Boland A, Boland R. 2008. Expression and localization of estrogen receptor α in the C2C12 murine skeletal muscle cell line. J Cell Biochem 104:1254-1273.

Miñano A, Xifró X, Pérez V, Barneda-Zahonero B, Saura CA, Rodríguez-Alvarez J. 2008. Estradiol facilitates neurite maintenance by a Src/Ras/ERK signalling pathway. Mol Cell Neurosci. 39(2):143-51.

Monks DA, O'Bryant EL, Jordan CL. 2004. Androgen receptor immunoreactivity in skeletal muscle: enrichment at the neuromuscular junction. J Comp Neurol. 473(1):59-72.

Murphy DD, Cole NB, Segal M. 1998. Brain-derived neurotrophic factor mediates estradiol-induced dendritic spine formation in hippocampal neurons. PNAS 95:11412-11417.

Niel L, Willemsen KR, Volante SN, Monks DA. 2008. Sexual dimorphism and androgen regulation of satellite cell population in differentiating rat levator ani muscle. Developmental Neurobiology 68(1):115-122.

Niel L, Shah AH, Lewis GA, Mo K, Chatterjee D, Fernando SM, Hong MH, Chang WY, Vollmayr P, Rosen J, Miner JN, Monks DA. 2009. Sexual differentiation of the spinal nucleus of the bulbocavernosus is not mediated solely by androgen receptors in muscle fibers. Endocrinology 150:3207-3213.

Nordeen EJ, Nordeen KW, Sengelaub DR, Arnold AP. 1985. Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. Science 229:671-673.

Nowacek AS, Sengelaub DR. 2006. Estrogenic support of motoneuron dendritic growth via the neuromuscular periphery in a sexually dimorphic motor system. J Neurobiol 66:962-976.

Osborne MC, Verhovshek T, Sengelaub, DR. 2007. Androgen regulates trkB expression in spinal motoneurons. J Neurosci Res. 85:303–309.

Ottem E, Beck L, Jordan C, Breedlove S. 2007. Androgen-dependent regulation of brain-derived neurotrophic factor and tyrosine kinase B in the sexually dimorphic spinal nucleus of the bulbocavernosus. Endocrinology 148:3655-3665.

Rand MN, Breedlove SM. 1995. Androgen alters the dendritic arbors of SNB motoneurons by acting upon their target muscles. J Neurosci 15:4408-4416.

Raouf S, Van Roo B, Sengelaub D. 2000. Adult plasticity in hormone-sensitive motoneuron morphology: Methodological/behavioral confounds. Horm Behav 38:210-221.

Rudolph LM, Sengelaub DR. 2013. Critical period for estrogen-dependent motoneuron dendrite growth is coincident with ERα expression in target musculature. Dev Neurobiol. 73(1):72-84.

Rudolph LM, Sengelaub DR. 2013. Castration-induced upregulation of muscle ERα supports estrogen sensitivity of motoneuron dendrites in a sexually dimorphic neuromuscular system. Dev Neurobiol. 73(12):921-35.

Saceda, M., Lippman, ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M, Martin MB. 1988. Regulation of the estrogen receptor in MCF-7 cells by estradiol. Mol Endocrinol. 2(12):1157-1162.

Sachs BD. 1982. Role of striated penile muscles in penile reflexes, copulation, and induction of pregnancy in the rat. J Reprod Fertil 66:433-443.

Sasahara K, Shikimi H, Haraguchi S, Sakamoto H, Honda S, Harada N, Tsutsui K. 2007. Mode of action and functional significance of estrogen-inducing dendritic growth, spinogenesis, and synaptogenesis in the developing purkinje cell. J Neurosci 27(28): 7408-7417.

Schrøder H. 1980. Organization of the motoneurons innervating the pelvic muscles of the rat. J Comp Neurol 192:567-587.

Shughrue PJ, Sar M, Stumpf WE. 1992. Progestin target cell distribution in forebrain and midbrain regions of the 8-day postnatal mouse brain. Endocrinology. 130(6):3650-3659.

Sohrabji F, Miranda R, Toran-Allerand C. 1994. Estrogen differentially regulates estrogen and nerve growth factor receptor mRNAs in adult sensory neurons. J Neurosci 14:459-471.

Sohrabji F, Miranda R, Toran-Allerand C. 1995. Identification of a putative estrogen response element in the gene coding for BDNF. PNAS USA 92:11110-11114.

Solum D, Handa R. 2002. Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus. J Neurosci 22:2650-2659.

Sumner BEH, Watson WE. 1971. Retraction and expansion of the dendritic tree of motor neurones of adult rats induced in vivo. Nature 233:273-275.

Swift-Gallant A, Monks DA. 2013. Androgen receptor expression in satellite cells of the neonatal levator ani of the rat. Dev Neurobiol DOI: 10.1002/dneu.22066.

Tanaka J, Arnold AP. 1993. An electrophysiological study of descending projections to the lumbar spinal cord in adult male rats. Exp Brain Res 96:117-124.

Taylor S, Widows MR, Sengelaub DR. 1995. Estrogenic influence and possible site of action in development of motoneuron morphology in a sexually dimorphic rat spinal nucleus. Soc Neurosci Abstr 21:40.

Tobin C, Joubert Y. 1991. Testosterone-induced development of the rat levator ani muscle. Dev Biol 146:131-138.

Velickovic K, Cvoro A, Srdic B, Stokic E, Markelic M, Golic I, Otasevic V, Stancic A, Jankovic A, Vucetic M, Buzadzic B, Korac B, Korac A. 2014. Expression and subcellular localization of estrogen receptors α and β in human fetal brown adipose tissue. J Clin Endocrinol Metab. 99(1):151-9.

Verhovshek T, Cai Y, Osborne MC, Sengelaub DR. 2010. Androgen regulates brain-derived neurotrophic factor in spinal motoneurons and their target musculature. Endocrinology 151:253-261.

Verhovshek T, Buckley KE, Sergent MA, Sengelaub DR. 2010. Testosterone metabolites differentially maintain adult morphology in a sexually dimorphic neuromuscular system. Dev Neurobiol 70:206-221.

Verhovshek T, Sengelaub DR. 2010. Trophic effects of brain-derived neurotrophic factor blockade in an androgen-sensitive neuromuscular system. Endocrinology 151:5337-5348.

Verhovshek T, Rudolph LM, Sengelaub DR. 2013. Brain-derived neurotrophic factor and androgen interactions in spinal neuromuscular systems. Neuroscience. 239:103-14.

Verhovshek T, Sengelaub DR. 2013. Androgen action at the target musculature regulates brain-derived neurotrophic factor protein in the spinal nucleus of the bulbocavernosus. Dev Neurobiol. 73(8):587-98.

Voyvodic JT. 1989. Peripheral target regulation of dendritic geometry in the rat superior cervical ganglion. J Neurosci 9:1997-2010.Verhovshek and Sengelaub, 2013

Walker DM, Kirson D, Perez LF, Gore AC. 2012. Molecular profiling of postnatal development of the hypothalamus in female and male rats. Biol Reprod. 87(6):129.

West M, Gunderson H. 1990. Unbiased stereological estimation of the number of neurons in the human hippocampus. J Comp Neurol 296:1-22.

Wiik A, Glenmark B, Ekman M, Esbjornsson-Liljedahl M, Johansson O, Bodin K, Enmark E, Jansson E. 2003. Oestrogen receptor beta is expressed in adult human skeletal muscle both at the mRNA and protein level. Acta Physiol Scand 179:381-387.

Xu J, Gingras K, Bengston L, DiMarco A, Forger N. 2001. Blockade of endogenous neurotrophic factors prevents the androgenic rescue of rat spinal motoneurons. J Neurosci 21:4366-4372.

Yang LY, Verhovshek T, Sengelaub DR. 2004. Brain-derived neurotrophic factor and androgen interact in the maintenance of dendritic morphology in a sexually dimorphic rat spinal nucleus. Endocrinology. 145(1):161-8.

Yang LY, Arnold AP. 2000. Interaction of BDNF and testosterone in the regulation of adult perineal motoneurons. J Neurobiol. 2000 44(3):308-19.

Yawo H. 1987. Changes in the dendritic geometry of mouse superior cervical ganglion cells following postganglionic axotomy. J Neurosci 7:3703-3711.

Appendix A

How to get a PhD

By: Rudy Rudolph

(hypothetical result)

Appendix B

Qualification Exam Limerick

By: Rudy Rudolph

The “female sex hormone” E2

Its membranous functions are new

It binds to ERs and is found in bars

In hop compounds inside your brew.

Lauren Marie Rudolph

Email: Lamarudo@indiana.edu

EDUCATION

2009-2014	PhD, Program in Neuroscience and Department of Psychological and Brain Sciences Indiana University, Bloomington, IN Advisor: Dr. Dale Sengelaub
2004-2008	Bachelor of Science, *cum laude*, Neuroscience and Psychology Washington & Lee University, Lexington, VA

PUBLICATIONS

Rudolph, L.M. & Sengelaub, D.R. (2013*)*. Castration-induced upregulation of muscle ERα supports estrogen sensitivity of motoneuron dendrites in a sexually dimorphic neuromuscular system. *Developmental Neurobiology.* 73(12): 921-935.

Verhovshek, T, **Rudolph, L.M.**, & Sengelaub, D.R. (2013). BDNF and androgen interactions in spinal neuromuscular systems. *Neuroscience*, 239(3): 103-114.

Rudolph, L.M. & Sengelaub, D.R. (2013). Critical period for estrogen-dependent motoneuron dendrite growth is coincident with ERα expression in target musculature. *Developmental Neurobiology.* 73(1): 72-84.

PUBLISHED ABSTRACTS/PRESENTATIONS/INVITED SEMINARS

Rudolph, L.M. & Sengelaub, D.R. (April 2014). Satellite cells in the target muscle are not critical for estrogen-dependent SNB motoneuron dendrite growth during early development. Poster presented at the Animal Behavior Conference, Bloomington, IN.

Rudolph, L.M. (April 11, 2014). What's critical about a critical period: The role of muscle ERα in motoneuron dendrite growth. Invited seminar presented to the Dept. of Neurobiology, University of California at Los Angeles.

Rudolph, L.M. & Sengelaub, D.R. (November 2013). Maintenance of ERα in the SNB target muscle maintains estrogen sensitivity of SNB dendrites outside the early postnatal period. Poster presented at the Society for Neuroscience annual meeting, San Diego, CA.

Rudolph, L.M. & Sengelaub, D.R. (August 2013). Mechanisms of behavior: motoneurons and the development, maintenance, and restoration of sex behavior. Talk co-presented at the Psychological and Brain Sciences-Biology MoB Retreat, Bloomington, IN.

Rudolph, L.M. & Sengelaub, D.R. (March 2013). Mechanisms of a critical period: Maintenance of muscle ERα permits estradiol-dependent dendrite growth in a sexually dimorphic neuromuscular system. Talk presented at the Animal Behavior Conference, Bloomington, IN.

Rudolph, L.M. & Sengelaub, D.R. (October 2012). Developmental expression of ERα in SNB target muscle is upregulated by castration and confers estrogen-sensitivity to SNB motoneuron dendrites. Poster presented at the Society for Neuroscience annual meeting, New Orleans, LA.

Rudolph, L.M. & Sengelaub, D.R. (April 2012). Defining a critical period: Developmental downregulation of muscle ERα is driven by gonadal steroids. Talk presented at the Animal Behavior Conference, Bloomington, IN.

Rudolph, L.M. & Sengelaub, D.R. (November 2011). Critical period for estrogen-dependent dendritic growth in a sexually dimorphic neuromuscular system coincides with presence of estrogen receptor in the target musculature. Poster presented at the Society for Neuroscience annual meeting, Washington, DC.

Rudolph, L.M. & Sengelaub, D.R. (October 2011). Transient expression of estrogen receptor in target musculature coincides with estrogenic support of motoneuron dendrite growth. Talk presented at the Psychology Mechanisms of Behavior area meeting, Bloomington, IN.

Rudolph, L.M. & Sengelaub, D.R. (April 2011). Transient expression of estrogen receptor in target musculature coincides with estrogenic support of motoneuron dendrite growth. Talk presented at the Animal Behavior Conference, Bloomington, IN.

Rudolph, L.M. & Sengelaub, D.R. (April 2011). Transient expression of estrogen receptor in target musculature coincides with estrogenic support of motoneuron dendrite growth. Talk presented at the Program in Neuroscience Colloquium, Bloomington, IN.

Rudolph, L.M. & Sengelaub, D.R. (November 2010). Transient expression of estrogen receptor in target musculature coincides with estrogenic support of motoneuron dendrite growth. Poster presented at the Society for Neuroscience annual meeting, San Diego, CA.

Reidy, B.L., Bradenham, B.P., Harrison, C.H., Leung, P.W., McClenon, C.C., **Rudolph, L.M.**, Stevens, V.M., & Stewart, R.E., (November 2008). Developmental alteration of hamster chorda tympani nerve terminal field morphology. Poster presented at the Society for Neuroscience annual meeting, Washington, DC.

HONORS & AWARDS

Graduate Professional Student Organization Travel Award, Fall 2013
Program in Neuroscience Travel Award, Fall 2013
Dept. of Psychological and Brain Sciences Travel Award, 2013
Indiana University Provost's Travel Award, 2013
Common Themes in Reproductive Diversity (CTRD) pre-doctoral trainee (NIH-CHD T32HD049336), 2013-2014
Graduate Professional Student Organization Research Award, 2013
CISAB graduate student fellowship, spring 2013
CISAB Travel Award, fall 2012
P.E.O. Scholar Award Nominee- EQ Chapter, 2012
Chroma/89 North Image Award, Society for Neuroscience Annual Meeting, 2012
Indiana University Neuroscience Fellowship, 2010-2011

MEMBERSHIPS

Indiana Academy of Science, student member since 2013
Society for Behavioral Neuroendocrinology, student member since 2013
Sigma Xi, elected 2012
Society for Neuroscience, student member since 2009
Center for Integrative Study of Animal Behavior, Bloomington, IN, student member since 2009

TEACHING EXPERIENCE

Fall 2013	Assistant Instructor
	Course: A502, Research/Professional Ethics in the Bio-Behavioral Sciences

Fall 2012	Assistant Instructor Course: N500, Neural Science I
Spring 2012	Lab Instructor Course: P211, Methods in Experimental Psychology
Fall 2011	Assistant Instructor Course: P201, An Intro to Neuroscience
Spring 2011	Assistant Instructor Course: P457, Stress Effects: Brain & Behavior
Fall 2010	Assistant Instructor Course: P346, Neuroscience

SERVICE

Center for Integrative Study of Animal Behavior, Animal Behavior Conference Satellite Symposium, “Sex: From codons to condoms, the mechanisms and consequences of sexual behavior” co-Organizer, 2013-2014

Department of Psychological and Brain Sciences Graduate Recruitment Student Advisory Committee, 2012-present

Center for Integrative Study of Animal Behavior, Animal Behavior Conference student planning committee, 2009-present